

# TNF-stimulated MAP kinase activation mediated by a Rho family GTPase signaling pathway

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**The biological response to tumor necrosis factor (TNF) involves activation of MAP kinases. Here we report a mechanism of MAP kinase activation by TNF that is mediated by the Rho GTPase family members Rac/Cdc42. This signaling pathway requires Src-dependent activation of the guanosine nucleotide exchange factor Vav, activation of Rac/Cdc42, and the engagement of the Rac/Cdc42 interaction site (CRIB motif) on mixed-lineage protein kinases (MLKs). We show that this pathway is essential for full MAP kinase activation during the response to TNF. Moreover, this MLK pathway contributes to inflammation in vivo.**

[*Keywords:* MAP kinase; MLK; mixed-lineage protein kinase; TNF]

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The tumor necrosis factor (TNF) signaling pathway is established to be a target for the development of effective strategies for the treatment of human inflammatory diseases (Feldmann 2002). Knowledge of the mechanism of TNF signaling is therefore important for the design of novel therapeutic approaches. Biological responses to TNF involve activation of signal transduction pathways, including MAP kinases and NF- $\kappa$ B (Karin 2005).

TNF recruits TRADD, TRAF2, and RIP1 to the cytoplasmic domain of TNF receptor 1 (Micheau and Tschoop 2003). This complex initiates signal transduction mediated by NF- $\kappa$ B. Activation of the NF- $\kappa$ B pathway requires ubiquitin-mediated activation of the protein kinase TAK1 (Skaug et al. 2009). Studies of *Tak1*<sup>-/-</sup> murine cells confirm that TAK1 is essential for TNF-induced activation of I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ) and the subsequent degradation of I $\kappa$ B $\alpha$ , a critical step in NF- $\kappa$ B signaling (Sato et al. 2005; Shim et al. 2005). The TAK1 protein kinase therefore plays a central role in TNF-mediated activation of NF- $\kappa$ B.

TNF-stimulated MAP kinase activation is also mediated by the TRADD/TRAF2/RIP1 complex that is recruited to the cytoplasmic domain of TNF receptor 1 (Micheau and Tschoop 2003). Several members of the MAP kinase kinase (MAP3K) group (e.g., ASK1 [Ichijo et al. 1997; Nishitoh et al. 1998; Tobiume et al. 2001], MEKK1 [Xia et al. 2000], mixed-lineage protein kinase 3 [MLK3] [Sathyanarayana et al. 2002; Chadee and Kyriakis 2004; Brancho et al. 2005; Korchnak et al. 2009; Sondarva et al. 2009], TAK1 [Sato et al. 2005; Shim et al. 2005], and TPL2 [Das et al. 2005]) have been implicated in TNF-induced MAP kinase activation. The relative contribution of these MAP3Ks to TNF-stimulated MAP kinase activation is unclear. The roles of individual MAP3Ks may reflect differences between TNF-responsive cells and/or differences between separate MAP kinase pathways. These MAP kinase pathways include the cJun N-terminal kinase (JNK), p38 MAP kinase, and extracellular signal-regulated kinases (ERKs). Each of these MAP kinases is activated by different MAP kinase kinase (MAP2K) isoforms. Thus, JNK activation requires MKK7 and cooperative signaling by MKK4 (Tournier et al. 2001), TNF-stimulated activation of p38 MAP kinase requires both MKK3 and MKK6 (Brancho et al. 2003), and ERK activation is mediated by MKK1 and MKK2 (Robinson and Cobb 1997). These

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pathways lead to ERK, JNK, and p38 MAP kinase activation by a dual phosphorylation (Thr and Tyr) mechanism.

It is established that TNF causes activation of Rho family GTPase proteins—including Rac/Cdc42 (Wojciak-Stothard et al. 1998; Kim et al. 1999; Puls et al. 1999; Hanna et al. 2001)—that may contribute to actin cytoskeleton reorganization (Wojciak-Stothard et al. 1998) and the production of reactive oxygen species (Kim et al. 2007; Yazdanpanah et al. 2009). The purpose of this study was to examine whether the TNF-stimulated Rac/Cdc42 pathway might contribute to MAP kinase activation (Coso et al. 1995; Minden et al. 1995). We report that TNF can activate a PTPN1/Src/Vav pathway that causes MLK-dependent activation of MAP kinases.

## Results

### *Requirement of the Rac/Cdc42 interaction site (CRIB motif) on MLK3 for TNF-stimulated MAP kinase activation*

Treatment of primary murine fibroblasts (MEFs) (Fig. 1A) and bone marrow-derived macrophages (BMDMs) (Fig. 1B) with TNF causes rapid activation of Rac and Cdc42. We examined whether this TNF-stimulated Rac/Cdc42 pathway might contribute to MAP kinase activation (Coso et al. 1995; Minden et al. 1995) by binding to the Rac/Cdc42 interaction site (CRIB motif) on MLK3 (Teramoto et al. 1996). To test this hypothesis, we investigated the effect of point mutations (Ile<sup>492</sup> and Ser<sup>493</sup> replaced with Ala) that disrupt the CRIB motif and prevent Rac/Cdc42-dependent activation of MLK3 (Bock et al. 2000). Mice with germline point mutations in the *MLK3* gene were established (*MLK3*<sup>ΔCRIB/ΔCRIB</sup> mice) and primary cultures of MEFs were prepared (Fig. 1C–F). We found similar TNF-stimulated MAP kinase activation in control (*MLK3*<sup>+/+</sup>) and mutant (*MLK3*<sup>ΔCRIB/ΔCRIB</sup>) MEFs (data not shown). This observation may reflect a redundant role of MLK3 in TNF-mediated signal transduction.

Gene expression analysis demonstrated that MEFs primarily express MLK2 and MLK3 (Supplemental Fig. S1). We therefore employed *MLK2*<sup>-/-</sup> mice (Supplemental Fig. S2), *MLK3*<sup>-/-</sup> mice (Brancho et al. 2005), and compound mutant *MLK2*<sup>-/-</sup> *MLK3*<sup>-/-</sup> mice (Supplemental Fig. S2) to prepare MEFs. Control studies demonstrated that MLK deficiency did not alter cell morphology or proliferation (Fig. 2). Deficiency of MLK2 or MLK3 caused only minor changes in TNF-stimulated MAP kinase activation (Supplemental Fig. S3), but TNF-stimulated MAP kinase activation in *MLK2*<sup>-/-</sup> *MLK3*<sup>-/-</sup> MEFs was markedly reduced (Fig. 1G). The reduced TNF-stimulated MAP kinase activation detected in *MLK2*<sup>-/-</sup> *MLK3*<sup>-/-</sup> MEFs was not associated with altered TNF-stimulated activation of IKKβ or degradation of IκBα (Fig. 1G). Thus, the MLK pathway (mediated by redundant functions of MLK2 and MLK3) is required for maximal TNF-stimulated MAP kinase activation, but is not required for TNF-stimulated activation of the NFκB pathway. This selective requirement of MLK2/3 for MAP kinase activation was also observed in studies of *MLK2*<sup>-/-</sup>

*MLK3*<sup>-/-</sup> BMDMs treated with TNF or lipopolysaccharide (LPS) (Fig. 1H).

MLK2 and MLK3 have partially redundant functions (Fig. 1G,H). We therefore re-examined the effect of the MLK3 CRIB mutation in a MLK2-deficient genetic background. This analysis demonstrated that TNF-stimulated MAP kinase activation in *MLK2*<sup>-/-</sup> *MLK3*<sup>ΔCRIB/ΔCRIB</sup> MEFs was markedly suppressed (Fig. 1I). In contrast, no difference in TNF-stimulated IκBα degradation was detected between wild-type and *MLK2*<sup>-/-</sup> *MLK3*<sup>ΔCRIB/ΔCRIB</sup> MEFs (Fig. 1I). These data confirm that the MLK pathway contributes to TNF-stimulated MAP kinase activation, but not TNF-stimulated NFκB activation.

To test whether Rac-mediated activation of MAP kinase is mediated by the MLK pathway, we expressed constitutively activated Rac in wild-type MEFs, *MLK2*<sup>-/-</sup> *MLK3*<sup>-/-</sup> MEFs, and *MLK2*<sup>-/-</sup> *MLK3*<sup>ΔCRIB/ΔCRIB</sup> MEFs (Supplemental Fig. S4). Activated Rac caused JNK activation in wild-type MEFs, but did not cause JNK activation in MEFs with MLK pathway defects. These data demonstrate that the MLK pathway is an essential mediator of Rac signaling.

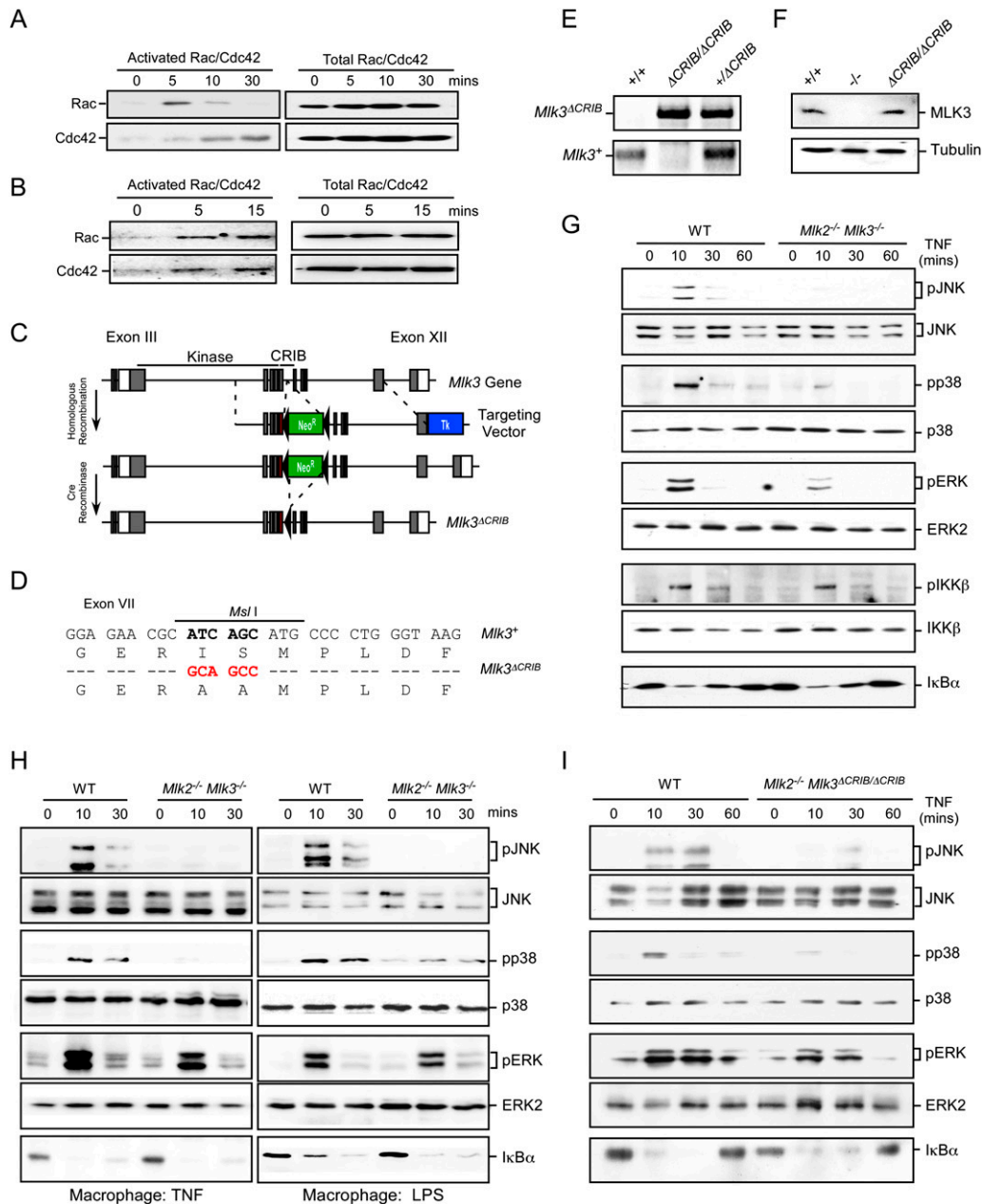
The low-level TNF-stimulated MAP kinase activation detected in *MLK2*<sup>-/-</sup> *MLK3*<sup>-/-</sup> MEFs and *MLK2*<sup>-/-</sup> *MLK3*<sup>ΔCRIB/ΔCRIB</sup> MEFs (Fig. 1G–I) may be mediated by TAK1. Indeed, TNF can cause partial MAP kinase activation in *TAK1*<sup>-/-</sup> MEFs (Fig. 3A), and RNAi-mediated knockdown of TAK1 in *MLK2*<sup>-/-</sup> *MLK3*<sup>-/-</sup> MEFs strongly reduced TNF-stimulated MAP kinase activation (Fig. 3B–D). Together, these data indicate that TNF-stimulated MAP kinase activation is mediated by the coordinated activation of at least two signaling pathways (MLK2/3 and TAK1).

### *Vav guanine nucleotide exchange factors mediate TNF signaling*

The suppression of TNF-stimulated MAP kinase activation caused by mutation of the MLK3 CRIB motif (Fig. 1) indicates that Rac/Cdc42 may mediate TNF-induced MAP kinase activation. However, the mechanism that accounts for TNF-stimulated Rac/Cdc42 activation is unclear. To identify TNF-regulated guanine nucleotide exchange factors (RhoGEFs), we isolated proteins that interact with a nucleotide-free form of Rac (Rac1<sup>Gly15Ala</sup>, a mimic of the guanine nucleotide transition state) from control and TNF-treated cells using affinity chromatography (Garcia-Mata et al. 2006). Vav was identified as a TNF-activated RhoGEF (Fig. 4A). In contrast, the RhoGEF isoforms GEFT and TIAM1 were not activated by TNF (Fig. 4A). To test whether Vav contributes to TNF-stimulated Rac activation, we examined Rac activation in wild-type and Vav1/2/3-deficient primary BMDMs (Fig. 4B). This analysis demonstrated that Vav contributes to TNF-stimulated Rac activation (Fig. 4B).

### *TNF causes tyrosine phosphorylation and activation of Vav*

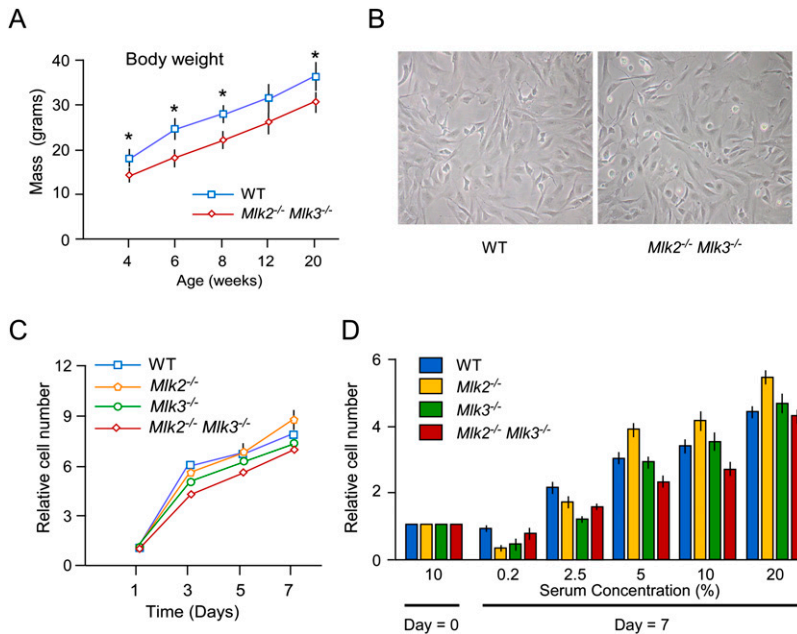
It is established that tyrosine phosphorylation represents a major mechanism of Vav regulation (Swat and Fujikawa 2005). Indeed, TNF caused increased Vav tyrosine phosphorylation (Fig. 4C). Candidate tyrosine kinases that might mediate this tyrosine phosphorylation include



**Figure 1.** TNF causes activation of a Rac/Cdc42–MLK signal transduction pathway. (A,B) MEFs (A) or BMDMs (B) were treated without or with 10 ng/mL TNF $\alpha$ . Activated (GTP-loaded) Rac/Cdc42 was isolated and quantitated by immunoblot analysis. The amount of Rac/Cdc42 in cell lysates was measured by immunoblot analysis. (C) The structure of the *Mik3* genomic locus and the targeting vector are illustrated. Homologous recombination causes the replacement of *Mik3* exon VII with a mutated form of exon VII together with the insertion of a floxed *Neo<sup>R</sup>* cassette. The floxed *Neo<sup>R</sup>* cassette was excised using *Cre* recombinase. (D) The point mutations in exon VII created by the targeting vector are illustrated. The nucleotide changes cause the elimination of an *Msp*I restriction site and the introduction of two amino acid substitutions (Ile492Ala and Ser493Ala) that disrupt the MLK3 CRIB domain. (E) Genomic DNA from wild-type, *Mik3<sup>+/-ΔCRIB</sup>*, and *Mik3<sup>ΔCRIB/ΔCRIB</sup>* mice was examined by PCR analysis to detect the wild-type and mutant *Mik3* alleles. (F) Extracts prepared from wild-type (WT), *Mik3<sup>-/-</sup>*, and *Mik3<sup>ΔCRIB/ΔCRIB</sup>* MEFs were examined by immunoblot analysis with antibodies to MLK3 and  $\alpha$ -Tubulin. (G–I) Wild-type and *Mik2<sup>-/-</sup> Mik3<sup>-/-</sup>* MEFs (G), wild-type and *Mik2<sup>-/-</sup> Mik3<sup>-/-</sup>* BMDMs (H), or wild-type and *Mik2<sup>-/-</sup> Mik3<sup>ΔCRIB/ΔCRIB</sup>* MEFs (I) were treated without or with 10 ng/mL TNF or 100 ng/mL LPS. MAP kinase and IKK $\beta$  activation and the degradation of I $\kappa$ B $\alpha$  were examined by immunoblot analysis.

members of the Src family (e.g., Src, Fyn, and Yes). We found that TNF did not cause Vav tyrosine phosphorylation in *Src<sup>-/-</sup> Fyn<sup>-/-</sup> Yes<sup>-/-</sup>* fibroblasts, but complementation analysis demonstrated that re-expression of Src was

sufficient to restore TNF-stimulated Vav tyrosine phosphorylation (Fig. 4D). Both Vav-deficient and Src/Fyn/Yes-deficient cells exhibited defects in TNF-stimulated activation of MAP kinases (Fig. 4E,F). Together, these data



**Figure 2.** The effect of MLK2 and MLK3 deficiency on growth. (A) The body mass of male wild-type (WT) and *Mik2<sup>-/-</sup> Mik3<sup>-/-</sup>* mice was measured at different time intervals (mean  $\pm$  SD;  $n = 8$ ). Statistically significant differences are indicated. (\*)  $P < 0.05$ . (B) Wild-type and *Mik2<sup>-/-</sup> Mik3<sup>-/-</sup>* MEFs were examined by phase-contrast microscopy. (C) The proliferation of wild-type, *Mik2<sup>-/-</sup>*, *Mik3<sup>-/-</sup>*, and *Mik2<sup>-/-</sup> Mik3<sup>-/-</sup>* MEFs during culture for 7 d was examined by plating  $2.5 \times 10^4$  MEFs in 11-mm wells using Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Relative cell numbers were measured by staining with crystal violet (mean  $\pm$  SD;  $n = 6$ ). No significant differences between wild-type and MLK-deficient MEFs were detected.  $P > 0.05$ . (D) MEFs were incubated for 0 or 7 d in Dulbecco's modified Eagle's medium supplemented with different amounts of fetal bovine serum. Relative cell numbers were measured by staining with crystal violet (mean  $\pm$  SD;  $n = 6$ ). No significant differences between wild-type and MLK-deficient MEFs were detected.  $P > 0.05$ .

demonstrate that a Src/Vav pathway contributes to TNF-stimulated MAP kinase activation.

#### Requirement of PTPN1 for TNF-stimulated Rac activation

Src family protein kinases have been previously implicated in TNF-stimulated MAP kinase activation mediated by TRAF2-associated tyrosine phosphatases (van Vliet et al. 2005). This pathway mediates TNF-stimulated MAP kinase activation, but not TNF-stimulated NF $\kappa$ B activation (van Vliet et al. 2005). The related tyrosine phosphatases PTPN1 and PTPN2 can cause opposite actions on Src family kinase activity by selectively dephosphorylating the inhibitory C-terminal site (PTPN1) or the activating T-loop site (PTPN2) of tyrosine phosphorylation (van Vliet et al. 2005). Increased PTPN1 and/or decreased PTPN2 function may therefore contribute to TNF-stimulated MAP kinase activation. We found that TNF caused decreased tyrosine phosphorylation of the C-terminal site of inhibitory tyrosine phosphorylation on Src (pY-527) in BMDMs isolated from control mice, but not from *Ptpn1<sup>-/-</sup>* mice (Fig. 5A). Moreover, TNF-stimulated Rac activation (Fig. 5B) and MAP kinase activation (Fig. 5C) was suppressed by PTPN1 deficiency. To confirm this observation, we examined the effect of selective small molecule inhibitors of PTPN1 and PTPN2. We found that inhibition of PTPN1 suppressed TNF-stimulated MAP kinase activation and that PTPN2 inhibition augmented TNF-stimulated MAP kinase activation (Fig. 5D). These data demonstrate that tyrosine phosphatases contribute to the regulation of TNF-stimulated MAP kinase activation.

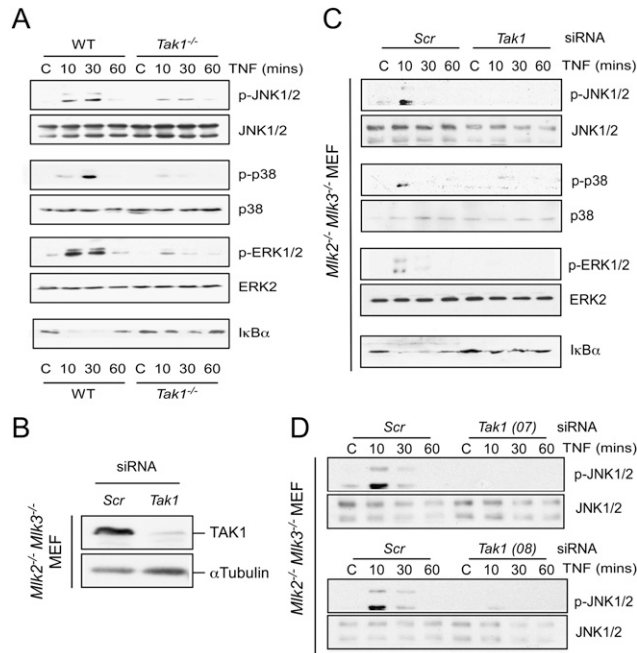
#### The MLK and TAK1 pathways mediate different TNF biological responses

The results of this study demonstrate that full activation of MAP kinases caused by TNF requires a Rac/Cdc42

signaling pathway that is mediated by Src-induced Vav activation and engagement of MLK protein kinases. To examine the contribution of this pathway to TNF signaling, we compared the response of control and MLK-deficient MEFs to treatment with TNF, including the expression of inflammatory cytokines and cell death. We found that TNF-treated *Mik2<sup>-/-</sup> Mik3<sup>-/-</sup>* MEFs secrete markedly less interleukin 6 (IL6) than wild-type cells (Fig. 6A), consistent with reduced MAP kinase activation in MLK-deficient cells (Fig. 1G,H). Previous studies have established that the ERK, JNK, and p38 MAP kinase pathways can contribute to *Il6* gene expression (Tuyt et al. 1999; Kim et al. 2004; Ventura et al. 2006), but increased IL6 expression caused by the Vav pathway is primarily mediated by JNK (Song et al. 1999). We found that *Mik2<sup>-/-</sup> Mik3<sup>-/-</sup>* MEFs (like wild-type MEFs) activate the NF- $\kappa$ B survival pathway (Fig. 1G) and exhibit resistance to the cytotoxic effects of TNF (Fig. 6B,C). In contrast, *Tak1<sup>-/-</sup>* cells were sensitive to TNF-mediated cytotoxicity (Fig. 6B,C) and failed to activate the NF- $\kappa$ B pathway (Fig. 3A). Together, these data demonstrate that the MLK and TAK1 pathways mediate different TNF-dependent cellular responses.

#### The MLK pathway contributes to inflammation in vivo

To test whether the MLK pathway can mediate inflammation, we examined the effect of MLK deficiency on the response of mice to endotoxin exposure. Treatment of wild-type mice with LPS caused increased expression of inflammatory cytokines (e.g., TNF $\alpha$ ) and chemokines (e.g., CCL3 and CCL5) in blood (Fig. 7A). These effects of LPS were suppressed in MLK-deficient mice (*Mik2<sup>-/-</sup> Mik3<sup>-/-</sup>*) and mice with MLK CRIB motif mutation (*Mik2<sup>-/-</sup> Mik3<sup>ACRIB/ACRIB</sup>*) (Fig. 7A). Control studies using in vitro cultures of BMDMs confirmed that the MLK pathway is required for LPS-stimulated cytokine



**Figure 3.** Residual MAP kinase activation in MLK-deficient MEFs is mediated by TAK1. (A) Wild-type (WT) and *Tak1*<sup>-/-</sup> MEFs were treated without or with TNF $\alpha$  (10 ng/mL). The activation of MAP kinases and the degradation of I $\kappa$ B $\alpha$  was examined by immunoblot analysis. (B,C) *Mlk2*<sup>-/-</sup> *Mlk3*<sup>-/-</sup> MEFs were transfected with control (Scr) siRNA or *Tak1* siRNA (72 h). Transfection assays were performed using DharmaFECT 3 reagent with ON-TARGET plus Nontargeting Pool D-001810-10 for control or ON-TARGET plus SMART pool L-040718-00 for *Tak1* (MAP3K7) siRNA (Thermo Scientific). (B) The expression of TAK1 and  $\alpha$ -Tubulin was examined by immunoblot analysis. (C) MAP kinase activation and the degradation of I $\kappa$ B $\alpha$  in response to treatment with TNF $\alpha$  (10 ng/mL) were examined by immunoblot analysis. (D) *Mlk2*<sup>-/-</sup> *Mlk3*<sup>-/-</sup> MEFs were transfected with scrambled (Scr) siRNA or *Tak1* siRNA (72 h). The MEFs were treated without or with 10 ng/mL TNF $\alpha$ . Protein extracts were examined by immunoblot analysis by probing with antibodies to JNK1/2 and phospho-JNK. The siRNA for *Tak1* were ON-TARGET plus siRNA J-040718-07 (top panel) and J-040718-08 (bottom panel).

and chemokine expression (Fig. 7B). Together, these data establish that the MLK pathway contributes to inflammation.

## Discussion

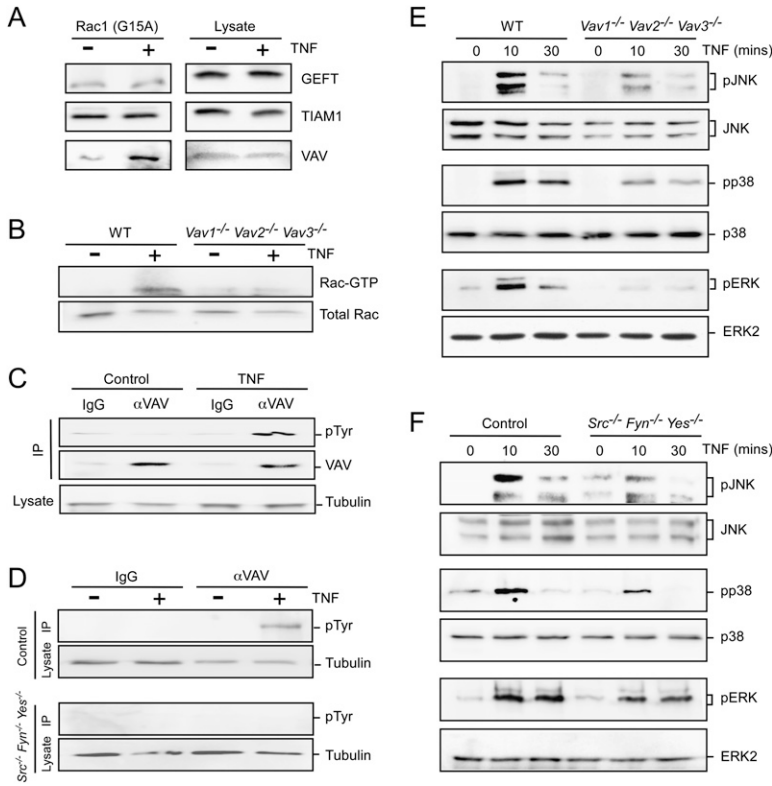
The biological response to TNF is mediated by the integrated actions of several signaling pathways. The NF- $\kappa$ B, MAP kinase, and caspase pathways are key mediators of the TNF response. TNF can also activate Rho family GTPases, including Rac/Cdc42 (Wojciak-Stothard et al. 1998; Kim et al. 1999; Puls et al. 1999; Hanna et al. 2001). Previous studies have established that Rac/Cdc42 may contribute to the effects of TNF on actin cytoskeleton reorganization (Wojciak-Stothard et al. 1998) and the production of reactive oxygen species (Kim et al. 2007; Yazdanpanah et al. 2009). Rac/Cdc42 therefore plays an

important role in the cellular response to TNF. Our study demonstrates that TNF-stimulated Rac/Cdc42 activation also contributes to MAP kinase regulation (Fig. 1).

The mechanism of TNF-stimulated Rac/Cdc42 activation requires Vav (Fig. 4B), a guanine nucleotide exchange factor that is activated by tyrosine phosphorylation (Crespo et al. 1997; Han et al. 1997) and can activate both Rac and Cdc42 (Abe et al. 2000; Liu and Burridge 2000). TNF causes tyrosine phosphorylation and activation of Vav by Src family protein kinases (Fig. 4C,D) that are activated by dephosphorylation on the inhibitory C-terminal site of tyrosine phosphorylation (Fig. 5A). Src activation requires the protein tyrosine phosphatase PTPN1 (Fig. 5A). The related phosphatase PTPN2 can oppose the activation of Src and functions to suppress TNF-stimulated MAP kinase activation (van Vliet et al. 2005). The balance of PTPN1 and PTPN2 can therefore determine the activation state of Src and, consequently, the extent of Vav-mediated Rac activation. PTPN2 interacts with TNF signaling complexes containing TRAF2 (van Vliet et al. 2005). The mechanism of TNF signaling may therefore be mediated by sequestration of the inhibitory phosphatase PTPN2, by recruitment of the activating phosphatase PTPN1, or by coordinated regulation of both phosphatases (activity and/or substrate access). Further studies are required to define this mechanism and to test whether Src activation might also require engagement of the Src SH3 domain. Indeed, it is possible that the Src-binding protein Sam68 (Fumagalli et al. 1994; Taylor and Shalloway 1994), which acts as an adapter in TNF receptor signaling complexes (Ramakrishnan and Baltimore 2011), links TNF signaling to the Src pathway. Nevertheless, the data reported here demonstrate that TNF can activate Rac by a PTPN1/Src/Vav pathway.

It is established that Rac/Cdc42 can cause MAP kinase activation (Coso et al. 1995; Minden et al. 1995). The effects of Rac/Cdc42 may be mediated by binding to the CRIB motif on MLK3 (Teramoto et al. 1996). We report that the functions of MLK3 are partially redundant with the related protein kinase MLK2 in MEFs (Fig. 1G) and macrophages (Fig. 1H). Compound gene disruption studies demonstrate that *Mlk2*<sup>-/-</sup> *Mlk3*<sup>-/-</sup> MEFs and macrophages exhibit a severe defect in TNF-stimulated MAP kinase activation (Fig. 1G,H). Moreover, the disrupted MLK CRIB motif in primary cells isolated from *Mlk2*<sup>-/-</sup> *Mlk3* <sup>$\Delta$ CRIB/ $\Delta$ CRIB</sup> mice also caused markedly reduced TNF-stimulated MAP kinase activation (Fig. 1I). These data indicate that MLK protein kinases mediate the effects of TNF-stimulated Rac/Cdc42 on MAP kinase activation.

The MLK protein kinases have been implicated in the activation of the NF- $\kappa$ B pathway (Hehner et al. 2000). However, we found no defects in TNF-stimulated IKK $\beta$  activation or I $\kappa$ B $\alpha$  degradation in *Mlk2*<sup>-/-</sup> *Mlk3*<sup>-/-</sup> MEFs (Fig. 1G). These data indicate that MLK protein kinases are not involved in the TNF-stimulated NF- $\kappa$ B pathway. Studies of *Tak1*<sup>-/-</sup> MEFs demonstrated that TAK1 deficiency completely ablated the TNF-stimulated NF- $\kappa$ B pathway, but caused only partial loss of TNF-stimulated MAP kinase activation (Fig. 3A). Together, these data indicate that TAK1 plays a key role in TNF-stimulated NF- $\kappa$ B

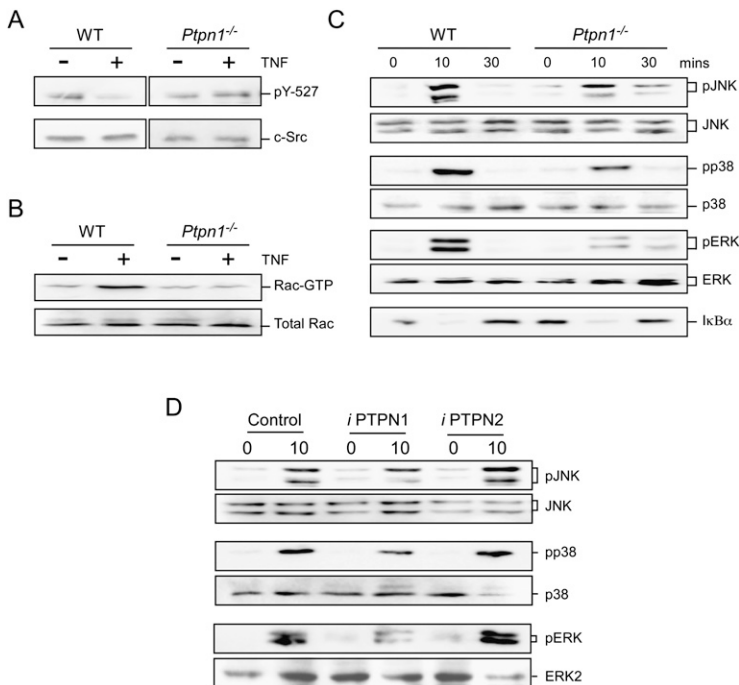


**Figure 4.** TNF-stimulated Rac activation is mediated by Src and Vav. (A) Lysates prepared from BMDMs treated without or with 10 ng/mL TNF (15 min) were incubated with immobilized Rac1<sup>G15A</sup>. Bound proteins and the cell lysates were examined by immunoblot analysis using antibodies to GEFT, TIAM1, and Vav. (B) Wild-type (WT) and *Vav1*<sup>-/-</sup> *Vav2*<sup>-/-</sup> *Vav3*<sup>-/-</sup> BMDMs were treated without or with 10 ng/mL TNF $\alpha$ . Activated (GTP-loaded) Rac was isolated and quantitated by immunoblot analysis. The amount of Rac in cell lysates was measured by immunoblot analysis. (C) Lysates prepared from wild-type BMDMs treated without or with TNF were examined by immunoprecipitation with a control antibody (IgG) or with an antibody to Vav. The immunoprecipitates were examined by immunoblot analysis using antibodies to phosphotyrosine (pTyr) and Vav. The cell lysates were examined by probing with an antibody to  $\alpha$ -Tubulin. (D) *Src*<sup>-/-</sup> *Fyn*<sup>-/-</sup> *Yes*<sup>-/-</sup> fibroblasts complemented with Src (Control) or without Src were treated without or with TNF $\alpha$ . Lysates were examined by immunoprecipitation with a control antibody (IgG) or with an antibody to Vav. The immunoprecipitates were examined by immunoblot analysis using antibodies to pTyr. The cell lysates were examined by probing with an antibody to  $\alpha$ -Tubulin. (E,F) Wild-type and *Vav1*<sup>-/-</sup> *Vav2*<sup>-/-</sup> *Vav3*<sup>-/-</sup> BMDMs (E) or Control and *Src*<sup>-/-</sup> *Fyn*<sup>-/-</sup> *Yes*<sup>-/-</sup> fibroblasts (F) were treated without or with 10 ng/mL TNF. MAP kinase activation was examined by immunoblot analysis.

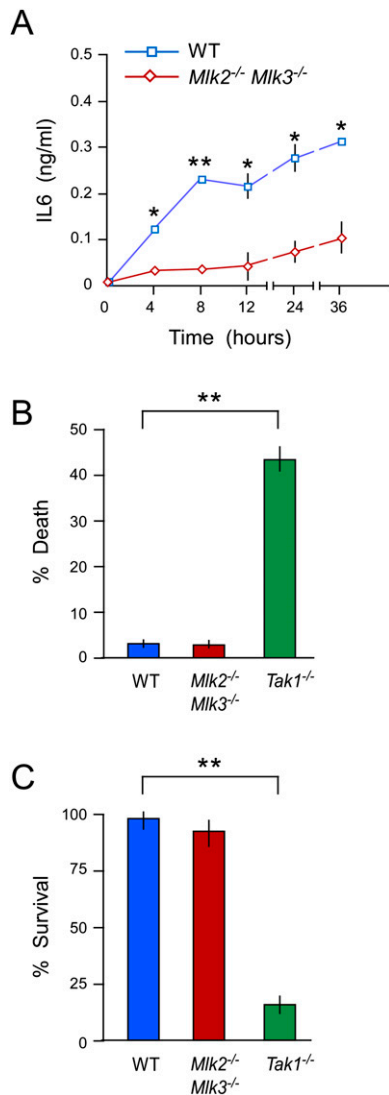
activation and that both TAK1 and MLK protein kinases contribute to TNF-stimulated MAP kinase activation.

The TAK1 and MLK pathways represent different signaling mechanisms employed by TNF receptor 1. Both

of these pathways provide an opportunity for therapeutic intervention that may be useful for the treatment of human inflammatory diseases. However, the effects of inhibition of these pathways are different because only



**Figure 5.** The protein tyrosine phosphatase PTPN1 contributes to TNF-stimulated MAP kinase activation. (A) Lysates prepared from wild-type (WT) and *Ptpn1*<sup>-/-</sup> BMDMs were treated without or with 10 ng/mL TNF $\alpha$  and examined by immunoblot analysis using antibodies to Src and pY<sup>527</sup> Src. (B) Wild-type and *Ptpn1*<sup>-/-</sup> BMDMs were treated without or with 10 ng/mL TNF (15 min). Activated (GTP-loaded) Rac1 was isolated and quantitated by immunoblot analysis. The amount of Rac1 in cell lysates was measured by immunoblot analysis. (C) Wild-type and *Ptpn1*<sup>-/-</sup> BMDMs were treated without or with 10 ng/mL TNF. MAP kinase activation and the degradation of I $\kappa$ B $\alpha$  was examined by immunoblot analysis. (D) BMDMs were treated with solvent or with drugs that selectively inhibit PTPN1 and PTPN2 (30 min). The cells were then treated with or without 10 ng/mL TNF $\alpha$  (10 min). Cell lysates were examined by immunoblot analysis using antibodies to phospho-JNK, JNK, phospho-p38, p38 $\alpha$ , phospho-ERK, and ERK2.



**Figure 6.** The MLK pathway contributes to TNF-stimulated signal transduction. (A) Wild-type (WT) and *Mik2*<sup>-/-</sup> *Mik3*<sup>-/-</sup> MEFs were treated with 10 ng/mL TNF $\alpha$ . The amount of IL6 in the culture medium was measured by ELISA (mean  $\pm$  SD;  $n = 6$ ). Statistically significant differences between wild-type and *Mik2*<sup>-/-</sup> *Mik3*<sup>-/-</sup> MEFs are indicated. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ . (B,C) Wild-type, *Mik2*<sup>-/-</sup> *Mik3*<sup>-/-</sup>, and *Tak1*<sup>-/-</sup> MEFs were treated with 10 ng/mL TNF (24 h). Cell death was examined by measuring the presence of lactate dehydrogenase in the medium (B), and cell viability was measured by staining with crystal violet (C). The data presented are the mean  $\pm$  SD ( $n = 4$ ). Statistically significant differences between wild-type and *Tak1*<sup>-/-</sup> MEFs are indicated. (\*\*)  $P < 0.01$ . No statistically significant differences between wild-type and *Mik2*<sup>-/-</sup> *Mik3*<sup>-/-</sup> MEFs were detected.

TAK1 inhibition causes decreased NF- $\kappa$ B-mediated survival signaling (Fig. 6B,C). The reduced TNF-mediated cytotoxicity caused by loss of MLK function, compared with loss of TAK1 function, may be a beneficial property of drugs that target MLK to inhibit TNF-stimulated inflammatory cytokine expression during disease progression in humans.

## Materials and methods

### Mice

C57BL/6J strain mice were obtained from The Jackson Laboratories. Mice with *Mlk3* (Brancho et al. 2005), *Ptpn1* (Klaman et al. 2000), *Tak1* (Shim et al. 2005), or *Vav1*, *Vav2*, and *Vav3* (Fujikawa et al. 2003) gene disruptions have been described previously.

Mice with germline mutations in exon VII of the *Mlk3* gene (*Mlk3* <sup>$\Delta$ CRIB</sup>) were constructed using homologous recombination in LC1 embryonic stem (ES) cells (albino C57BL/6J genetic background). The targeting vector was constructed using a mouse strain C57BL/6J genomic BAC clone containing the *Mlk3* gene, a floxed *Neo*<sup>R</sup> cassette, and a thymidine kinase cassette (Fig. 1C). The ES cells were electroporated and selected with 200  $\mu$ g/mL G418 and 2  $\mu$ M gangcyclovir. Correctly targeted ES cell clones were injected into C57BL/6J blastocysts to create chimeric mice that transmitted the mutated *Mlk3* allele through the germline. The floxed *Neo*<sup>R</sup> cassette was excised using *Cre* recombinase.

*Mlk2*<sup>-/-</sup> gene disruption by homologous recombination using 129/SvJ strain ES cells was used to create a deletion (exon I to exon VI) within the *Mlk2* gene (Supplemental Fig. S2). Correctly targeted ES cell clones were employed to create chimeric mice that transmitted the mutated *Mlk2* allele through the germline (D.S. Dorow, Peter MacCallum Cancer Institute). The *Mlk2*<sup>-/-</sup> mice were backcrossed to the C57BL/6J strain (10 generations).

The mice were housed in a facility accredited by the American Association for Laboratory Animal Care. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

### Genotype analysis

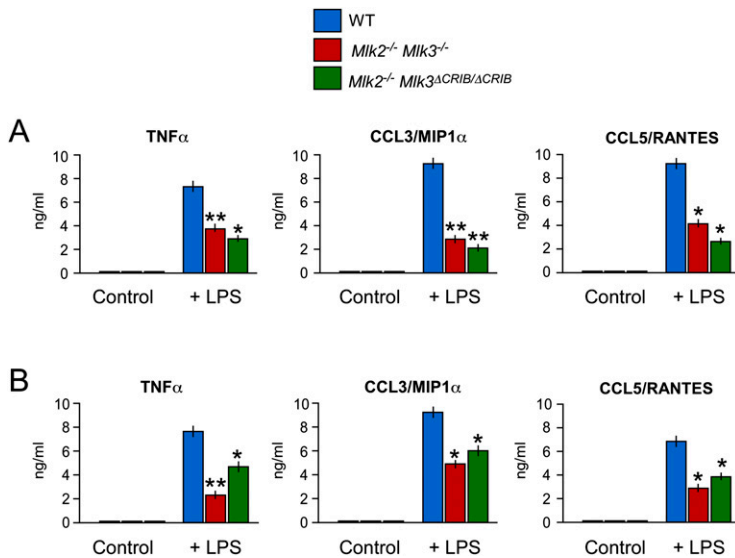
Wild-type (500-base-pair [bp]) and disrupted (600-bp) alleles of *Mlk2* were detected by PCR using the amplimers 5'-CCTGG TTCTACTGGGACAACAG-3', 5'-GTCACATCCACTTTCC TGGGC-3', and 5'-CGCCTTCTATCGCCTTCTTGAC-3'. Wild-type and disrupted alleles of *Mlk3* (Brancho et al. 2005), *Ptpn1* (Klaman et al. 2000), *Tak1* (Shim et al. 2005), or *Vav1/2/3* (Fujikawa et al. 2003) were detected by PCR analysis using genomic DNA as the template. The *Mlk3* <sup>$\Delta$ CRIB</sup> allele (160 bp) was detected by PCR amplification of genomic DNA using the amplimers 5'-CCCAACCCCAAATAAGC-3', and 5'-CGGAGAACGCA TCAGC-3'; the corresponding wild-type allele (160 bp) was detected using the amplimers 5'-CCCAACCCCAAATAAGC-3' and 5'-CGGAGAACGCGCAGCC-3'.

### Cell culture

BMDMs were prepared (Das et al. 2009) and cultured in Dulbecco's modified Eagle's medium supplemented with 30% L929 supernatant (source of M-CSF), 20% fetal bovine serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 2 mM L-glutamine (Invitrogen).

*Src*<sup>-/-</sup> *Fyn*<sup>-/-</sup> *Yes*<sup>-/-</sup> fibroblasts complemented without and with *Src* (Klinghoffer et al. 1999) were obtained from the American Type Culture Collection (no. CRL2459 and no. CRL2498) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 2 mM L-glutamine (Invitrogen).

Embryonic day 13.5 (E13.5) primary MEFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 2 mM L-glutamine (Invitrogen). Retroviral transduction assays (Lamb et al. 2003) were performed using the vectors pBABE-puro and pBABE-Rac1<sup>Q61L</sup>-puro. Transfection assays were performed



**Figure 7.** The MLK pathway contributes to LPS-mediated inflammation. (A) Wild-type (WT) mice, *Mlk2*<sup>-/-</sup> *Mlk3*<sup>-/-</sup> mice, and *Mlk2*<sup>-/-</sup> *Mlk3* $\Delta$ CRIB/ $\Delta$ CRIB mice were treated without and with 20 mg/kg LPS by intraperitoneal injection (2 h). The amount of TNF $\alpha$ , CCL3/MIP $\alpha$ , and CCL5/RANTES in the serum was measured by ELISA (mean  $\pm$  SD;  $n = 8$ ). Statistically significant differences between wild-type and MLK-deficient mice are indicated. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ . (B) Primary BMDMs were isolated from wild-type mice, *Mlk2*<sup>-/-</sup> *Mlk3*<sup>-/-</sup> mice, and *Mlk2*<sup>-/-</sup> *Mlk3* $\Delta$ CRIB/ $\Delta$ CRIB mice. The BMDMs were treated without and with 100 ng/mL LPS (24 h). The amount of TNF $\alpha$ , CCL3/MIP $\alpha$ , and CCL5/RANTES in the culture medium was measured by ELISA (mean  $\pm$  SD;  $n = 6$ ). Statistically significant differences between wild-type and MLK-deficient BMDMs are indicated. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ .

using DharmaFECT 3 reagent with ON-TARGET plus Nontargeting Pool D-001810-10 for control or ON-TARGET plus SMART pool L-040718-00 for *Tak1* (MAP3K7) siRNA (Thermo Scientific). Single siRNA for *Tak1* were ON-TARGET plus siRNA J-040718-07 and ON-TARGET plus siRNA J-040718-08. Cells were harvested at 72 h post-transfection. Proliferation assays and cell viability assays were performed by staining cells with crystal violet (Tournier et al. 2000). Cell death was examined by measurement of lactate dehydrogenase release using a Cytotoxicity Detection kit (Roche Applied Science).

The cells were treated with 10 ng/mL TNF $\alpha$  (R&D Systems) or 100 ng/mL LPS (Sigma). The cells were pretreated (30 min) without or with an inhibitor of PTPN1 (200 nM) or PTPN2 (20 nM) (Sun et al. 2003; Zhang et al. 2009).

#### RNA analysis

The expression of mRNA was examined by quantitative PCR analysis using a 7500 Fast Real-Time PCR machine (Applied Biosystems). TaqMan assays were used to quantitate *Mlk1* (Mm00616834\_m1), *Mlk2* (Mm01175535\_m1), *Mlk3* (Mm01233534\_m1), *Mlk4* (Mm00524049\_m1), *Tak1* (Mm00554514\_m1), *Tnfr1* (Mm01182929\_m1), *Tnfr2* (Mm00441889\_m1), and *Gapdh* (4352339E-0904021) mRNA (Applied Biosystems). Standard curves were constructed using the threshold cycle (Ct) values for each template dilution plotted as a function of the logarithm of the amount of input template. The number of mRNA copies for each gene sample combination was calculated using the slope of the standard curve. To obtain a normalized abundance, copy numbers were corrected for the amount of *Gapdh* mRNA in each sample.

#### Immunoblot analysis

Cell extracts were prepared using Triton lysis buffer (20 mM Tris at pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL aprotinin and leupeptin). The lysates (50  $\mu$ g of protein) were examined by protein immunoblot analysis. Immunocomplexes were detected by enhanced chemiluminescence (NEN). Primary antibodies were obtained from Cell Signaling (Cdc42, phospho-IKK $\beta$ , IKK $\beta$ ,

MLK3, phospho-ERK1/2, phospho-JNK1/2, p38 $\alpha$ , phospho-p38, Src, pY<sup>527</sup>-Src, Vav1/2/3, Rac1/2/3, and I $\kappa$ B $\alpha$ ), BD Pharmingen (JNK1/2), Millipore (Rac1, Vav, and phosphotyrosine), Santa Cruz Biotechnologies (ERK2, TAK1, and TIAM1), Sigma ( $\alpha$ -Tubulin), and Proteintech Group (GEFT). The polyclonal antibody to MLK2 was purified from serum obtained from rabbits immunized with a peptide that corresponds to MLK2 amino acids 898–911 (CISPPSRPDTPEPSPG-amide) conjugated to keyhole limpet hemocyanin (Yenzym).

#### Immunoprecipitation

Cell extracts were prepared using lysis buffer (150 mM NaCl, 50 mM Tris at pH 8.0, 5 mM EDTA, 1% NP-40, 1 mM PMSF) and incubated (5 h at 4°C) with 10  $\mu$ g of control nonimmune rabbit IgG (Santa Cruz Biotechnologies) or 10  $\mu$ g of rabbit antibodies to Vav (Millipore) and Vav1/2/3 (Cell Signaling). Immunocomplexes isolated using Protein G Sepharose were washed (five times) with lysis buffer.

#### Cytokine and chemokine analysis

Cytokines in cell culture medium were measured by multiplexed ELISA using a Luminex 200 machine (Millipore) and a serum mouse cytokine kit (Millipore).

#### Rac/Cdc42 activation

Assays were performed with the Rac/Cdc42 Activation Assay kit using the manufacturer's recommendations (Millipore). Cdc42 was detected by immunoblot analysis (Cell Signaling). Rac was detected by immunoblot analysis using an antibody to Rac1 (Millipore) for studies of MEFs and an antibody to Rac1/2/3 (Cell Signaling) for BMDM studies.

#### Analysis of RhoGEF proteins

TNF-activated RhoGEFs were identified by affinity chromatography using a nucleotide-free transition state mimic of Rac1 (Garcia-Mata et al. 2006). Cell extracts prepared using lysis buffer (150 mM NaCl, 50 mM Tris at pH 8.0, 5 mM EDTA, 1% NP-40, 1 mM PMSF) were incubated with GST-Rac1<sup>G15A</sup> immobilized on



GSH-Sepharose for 1 h at 4°C. The GSH-Sepharose was washed, and bound proteins were examined by immunoblot analysis.

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