

TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo

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TGF- β -activated kinase 1 (TAK1), a member of the MAPKKK family, is thought to be a key modulator of the inducible transcription factors NF- κ B and AP-1 and, therefore, plays a crucial role in regulating the genes that mediate inflammation. Although *in vitro* biochemical studies have revealed the existence of a TAK1 complex, which includes TAK1 and the adapter proteins TAB1 and TAB2, it remains unclear which members of this complex are essential for signaling. To analyze the function of TAK1 *in vivo*, we have deleted the *Tak1* gene in mice, with the resulting phenotype being early embryonic lethality. Using embryonic fibroblasts lacking TAK1, TAB1, or TAB2, we have found that TNFR1, IL-1R, TLR3, and TLR4-mediated NF- κ B and AP-1 activation are severely impaired in *Tak1^{tm/m}* cells, but they are normal in *Tab1^{-/-}* and *Tab2^{-/-}* cells. In addition, *Tak1^{tm/m}* cells are highly sensitive to TNF-induced apoptosis. TAK1 mediates IKK activation in TNF- α and IL-1 signaling pathways, where it functions downstream of RIP1-TRAF2 and MyD88-IRAK1-TRAF6, respectively. However, TAK1 is not required for NF- κ B activation through the alternative pathway following LT- β signaling. In the TGF- β signaling pathway, TAK1 deletion leads to impaired NF- κ B and c-Jun N-terminal kinase (JNK) activation without impacting Smad2 activation or TGF- β -induced gene expression. Therefore, our studies suggest that TAK1 acts as an upstream activating kinase for IKK β and JNK, but not IKK α , revealing an unexpectedly specific role of TAK1 in inflammatory signaling pathways.

[*Keywords:* JNK; NF- κ B; TAB1; TAB2; TAK1; TGF- β]

Supplemental material is available at <http://www.genesdev.org>.

Received August 2, 2005; revised version accepted September 19, 2005.

The NF- κ B and c-Jun N-terminal kinase (JNK) pathways play critical roles in the immune response and protect cells from apoptosis induced by various stressors and inflammatory cytokines. Due to their roles in various disease states and their potential as therapeutic targets, the signaling pathways activated by the inflammatory cytokines TNF- α and IL-1 have been intensely studied. TNF- α induces trimerization of its cognate receptor (TNFR1) and binding of the adaptor proteins TRADD, TRAF2, TRAF5, and RIP1, which leads to recruitment of the IKK complex and JNK to the receptor complex (Song

et al. 1997). Activated IKK triggers degradation of I κ B α and subsequent translocation of NF- κ B to the nucleus, leading to transcription of a subset of TNF-regulated genes (Hayden and Ghosh 2004). Once activated, JNK translocates to the nucleus, where it regulates the activity of the AP-1 transcription factor complex (c-Jun and ATF-2) (Chen and Goeddel 2002). In the IL-1 signaling pathway, interaction of IL-1 with its cognate receptor (IL-1R) and IL-1R accessory protein (IL-1Racp) forms an activated receptor complex that recruits the adaptor proteins MyD88, IRAK1, IRAK4, and TRAF6. Phosphorylation and degradation of IRAK1 induces ubiquitination of TRAF6 and release of the adaptor protein complex into the cytoplasm to activate downstream kinases, including IKK and JNK (Deng et al. 2000; Takaesu et al. 2001; Jiang et al. 2002).

TGF- β -activated kinase 1 (TAK1), a member of the

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Article published online ahead of print. Article and publication date are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1360605>.

MAPKKK family, was originally identified as a key regulator of MAP kinase activation in TGF- β -induced signaling pathways (Yamaguchi et al. 1995). During *Xenopus* embryonic development, TAK1 is involved in mesoderm induction and patterning mediated by bone morphogenetic protein (BMP), a TGF- β family ligand (Shibuya et al. 1998). Recent studies have reported that *Drosophila* TAK1 is required for both JNK and NF- κ B (Relish) activation in response to immune challenge by gram-negative bacteria infection and that IMD signaling is impaired in TAK1^{-/-} flies (Vidal et al. 2001; Boutros et al. 2002; Silverman et al. 2003; Park et al. 2004). In mammals, in vitro and overexpression studies suggest TAK1 is involved in TNFR1 and IL-1R/TLR-mediated signaling pathways upstream of IKK and JNK/p38 MAP kinases (Takaesu et al. 2003). Two mammalian TAK1 adaptor proteins, TAB1 and TAB2, were isolated as TAK1-interacting proteins by yeast two-hybrid screening. TAB1 interacts constitutively with TAK1 and induces TAK1 kinase activity when overexpressed (Shibuya et al. 1996). Following IL-1 stimulation, TAB2 translocates from the cell membrane to the cytosol and links TAK1 with TRAF6, thereby mediating TAK1 activation (Takaesu et al. 2000). In vitro biochemical studies have demonstrated that TRIKA1 (a complex of Ubc13 and Uev1A) and TRIKA2 (the complex of TAK1, TAB1, and TAB2) are key signal-transducing complexes that activate IKK and MKK6 in a manner dependent upon ubiquitination of TRAF6 (Wang et al. 2001). However, the physiological roles of mammalian TAK1, TAB1, and TAB2 in inflammatory signaling in vivo remain to be definitively established. TAB1-deficient mouse embryonic fibroblasts (MEFs) have been generated; however, TAB1 function has not been tested in TNFR1 and IL-1R/TLR-mediated signaling (Komatsu et al. 2002). Surprisingly, TAB2-deficient MEFs are capable of activating IKK and JNK normally in response to TNF- α or IL-1 (Sanjo et al. 2003). Thus, available genetic evidence has not yet recapitulated the predicted role for the TAK1 complex in mammalian cells in signaling to NF- κ B and AP-1.

Therefore, to definitively establish the function of TAK1 in vivo, we have generated mutant *Tak1^{m/m}* mice, which have an embryonic lethal phenotype that is distinct from that of TAB1 and TAB2-deficient mice. To circumvent the difficulty posed by this early lethality, we have investigated signaling to JNK and NF- κ B in embryonic fibroblasts derived from these mice. Unlike *Tab1^{-/-}* and *Tab2^{-/-}* cells, *Tak1^{m/m}* cells exhibit dramatically impaired NF- κ B and JNK activation through TNFR1, IL-1R, and TLR3, and are highly sensitive to TNF- α -induced apoptosis. TAK1, but not TAB1 or TAB2, is an essential signal transducer from the RIP-TRAF2 and MyD88-IRAK1-TRAF6 complexes to the IKK complex. Although TAK1 is essential for TLR4-induced AP-1 activation, the requirement for TAK1 in NF- κ B activation by LPS appears less complete. However, TAK1 is not required for the induction of p100 processing or NF- κ B activation by LT- β , suggesting differential requirements for activation of the alternative IKK α -mediated

NF- κ B pathway. In *Tak1^{m/m}* cells, Smad2 activation and gene expression following TGF- β are normal, whereas NF- κ B and JNK activation are impaired. Therefore, this in vivo analysis of the mammalian TAK1 complex establishes the specific requirement for TAK1, but not TAB1 or TAB2, as an upstream activator of IKK β and JNK in multiple signaling pathways to AP-1 and NF- κ B.

Results

Generation of *Tak1* mutant embryos

To explore the biological function of TAK1 in vivo, we generated TAK1-deficient (*Tak1^{m/m}*) mice by microinjecting gene trap-mutated *Tak1* embryonic stem cells (ES) into blastocysts of C57BL/6 mice (Fig. 1A; Stryke et al. 2003; Austin et al. 2004; Skarnes et al. 2004). To confirm the generation of embryos from the gene trap-mutated *Tak1* ES cells, β -galactosidase expression was analyzed with embryonic day 9.5 (E9.5) wild-type (*Tak1^{+/+}*) and heterozygous embryos (*Tak1^{+/m}*) (Fig. 1B). PCR genotyping demonstrated that β -galactosidase was expressed in heterozygous (*Tak1^{+/m}*), but not wild-type embryos (*Tak1^{+/+}*). Immunoblotting analysis with anti-TAK1 antibody confirmed the expression of TAK1 in wild-type (*Tak1^{+/+}*) and heterozygous (*Tak1^{+/m}*), but not homozygous mutant (*Tak1^{m/m}*) embryos (Fig. 1C).

Abnormal development of neural tube in *Tak1* mutant embryos

We next examined the genotypes of newborn offspring following intercrossing of *Tak1* heterozygous parents. No homozygous mutant mice (*Tak1^{m/m}*) were scored, suggesting that disruption of the *Tak1* gene causes an embryonic lethality (Table 1). All of the embryos between E8.5 and E9.5 were scored according to Mendelian expectations, whereas at E11.5 all homozygous mutant embryos were dead and being resorbed, and empty deciduas were frequently observed (Table 1). Thus, these results suggest that *Tak1* mutant embryos begin to die around E10. Most wild-type embryos and mutant littermates did not exhibit any gross morphological difference until E9.0, after which the mutant embryos displayed severe developmental abnormalities in the neural tube (Fig. 1D). At E9.5, most of the mutant embryos were smaller than wild-type and heterozygous embryos, and at E10 and E10.5, the head fold and neural tube were wavy and not closed in the mutant embryos (Fig. 1E). After E10.5, the embryonic structures were resorbed in the *Tak1* mutants. Interestingly, the mutant phenotype of *Tak1* embryos is substantially different from *Tab1* (abnormal cardiovascular and pulmonary morphogenesis) and *Tab2* (liver degeneration and apoptosis) mutant embryos (Table 2; Komatsu et al. 2002; Sanjo et al. 2003). The phenotypes of the mutant embryos suggest that the TAK1 complex does not only function as a single unit but, instead, that each component has a distinct role during development.

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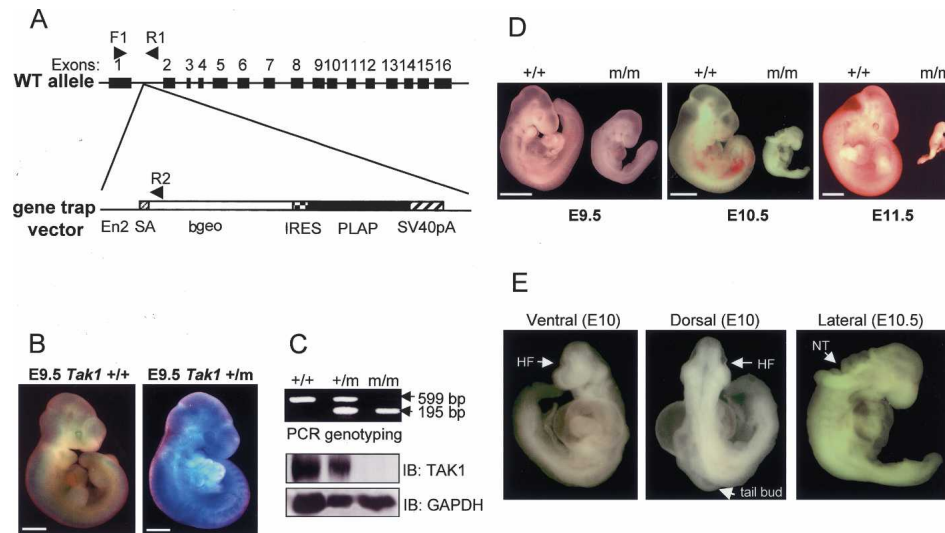


Figure 1. Targeted disruption of the *Tak1* gene. (A) Schematic drawing of the *Tak1* gene-trapping strategy. The position of primers (F1, R1, and R2) used in PCR genotyping are indicated by arrowheads. (En2) engrailed 2 gene; (βgeo) a fusion protein between β-galactosidase and neomycin phosphotransferase; (IRES) internal ribosomal entry site; (PLAP) human placental alkaline phosphatase; (SV40 pA) SV40 polyadenylation signal. (B) β-galactosidase staining of E9.5 wild-type (*Tak1*^{+/+}) and heterozygous embryos (*Tak1*^{+/m}). Bar, 100 μm. (C) PCR genotyping and immunoblotting analysis of TAK1 in E10 wild-type (+/+), heterozygous (+/m), and homozygous (m/m) mutant embryos. The size of PCR fragments is 599 base pairs (bp) and 195 bp for wild-type and insertional mutant alleles, respectively. (D) Lateral view of the wild type (+/+) and *Tak1* mutant (m/m) embryos at E9.5, E10.5, and E11.5. (E) Ventral (left) and Dorsal (middle) view of E10 *Tak1* mutant embryos and lateral view of E10.5 *Tak1* mutant embryos (right). Whole embryos were used for PCR genotyping. (HF) Head fold; (NT) neural tube.

TAK1, but not *TAB1* or *TAB2*, is required for *NF-κB* and *JNK* activation in *TNF-α* and *IL-1* signaling pathways

Despite numerous attempts, we were unable to culture embryonic fibroblasts from *TAK1*^{m/m} embryos to obtain sufficient cells for study. Therefore, to analyze the function of TAK1 in inflammatory signaling pathways, we generated immortalized embryonic fibroblasts derived from E9.75 wild-type (*Tak1*^{+/+}), heterozygous (*Tak1*^{+/m}), and homozygous mutant (*Tak1*^{m/m}) embryos by stably transfecting linearized SV40 large T antigen plasmid (Kobayashi et al. 2005). Immunoblotting analysis with anti-TAK1 antibody showed that endogenous TAK1 is expressed in *Tak1*^{+/+} and *Tak1*^{+/m} cells, but not *Tak1*^{m/m} cells (data not shown).

To address the hypothesis that the TAK1 complex, including TAK1, TAB1, and TAB2 is required for *NF-κB* and *JNK* activation in response to inflammatory cytokines, we tested the requirement for each member of the TAK1 complex in *TNF-α* and *IL-1* signaling pathways by luciferase analysis. Cells were transfected with *NF-κB* and AP-1-responsive luciferase reporters and stimulated with *TNF-α* or *IL-1* (Fig. 2A,B). Following activation by *TNF-α* and *IL-1*, *Tab1*^{-/-} and *Tab2*^{-/-} cells exhibit wild-type or greater levels of *NF-κB* and AP-1, whereas both *NF-κB* and AP-1 activation were significantly decreased in *Tak1*^{m/m} cells. This is consistent with in vitro experiments that indicate overexpression of kinase-inactive TAK1 mutant (TAK1-DN) inhibited both *TNF-α* and *IL-1*-induced *NF-κB* activation in a dose-dependent manner (Supplementary Fig. S1A,B). Interestingly, *Tab2*^{-/-} cells

show a small, but reproducible increase in signaling to both AP-1 and *NF-κB*. We also examined *TNF-α* and *IL-1*-induced *NF-κB* and *JNK* activation by immunoblotting with antibodies specific for *IκBα* and phospho-*JNK1/2*, respectively (Fig. 2C,D). In wild-type, *Tab1*^{-/-}, and *Tab2*^{-/-} cells, *IκBα* degradation and *JNK* phosphorylation occurs normally and peaks at 15 min in response to *TNF-α* and *IL-1*. In contrast, in the absence of TAK1 *IκBα* degradation and *JNK* phosphorylation following *TNF-α* and *IL-1* stimulation is markedly decreased. Furthermore, phosphorylation and nuclear translocation of c-Jun following *TNF-α* or *IL-1* stimulation occurs normally in wild-type cells, whereas *Tak1*^{m/m} cells exhibit impaired c-Jun activation (Fig. 2E). We next used electrophoretic mobility gel-shift assay (EMSA) to analyze *NF-κB* DNA-binding activity following *TNF-α* and *IL-1* stimulation (Fig. 2F,G). Wild-type, *Tab1*^{-/-}, and *Tab2*^{-/-} cells exhibited similar *NF-κB* DNA-binding activities in

Table 1. Genotype of offspring derived from *Tak1* heterozygous parents

	<i>Tak1</i> ^{+/+}	<i>Tak1</i> ^{+/-}	<i>Tak1</i> ^{-/-}
E8.5	27	52	22
E9.5	17	34	13
E10.5	13	28	9 (3) ^a
E11.5	16	38	5 (5)
E12.5	21	53	1 (1)
E13.5	15	37	0
Newborn	46	81	0

^aThe number of dead embryos is indicated in parentheses.

Table 2. Comparison of phenotypes of *Tak1*, *Tab1*, and *Tab2* mutant embryos

<i>Tak1</i> ^{-/-}	<i>Tab1</i> ^{-/-b}	<i>Tab2</i> ^{-/-c}
Ubiquitous expression at E14.5 (strong expression in nervous system, kidney, gut, testis, liver, lung, and pancreas) ^a	Ubiquitous expression through embryogenesis	Ubiquitous expression (strong expression in heart and liver)
Embryonic lethality around E10	Embryonic lethality at late stage of gestation	Embryonic lethality around E12.5
Neural fold dysmorphogenesis	Cardiovascular and lung dysmorphogenesis	Liver degeneration and apoptosis

^aJadrich et al. 2003.^bKomatsu et al. 2002.^cSanjo et al. 2003.

response to TNF- α and IL-1, whereas in *Tak1*^{m/m} cells, TNF- α -induced binding activity was completely abolished and IL-1-induced activity was partially reduced. In agreement with the luciferase analysis, *Tab2*^{-/-} cells showed higher IL-1-induced NF- κ B DNA-binding activity than wild-type or *Tab1*^{-/-} cells, implying that TAB2 negatively regulates NF- κ B activation. Therefore, these results provide genetic evidence of a requirement for TAK1, but not TAB1 or TAB2, in NF- κ B and JNK activation in the TNF- α and IL-1 signaling pathways.

TAK1, but not TAB1 or TAB2, is required for NF- κ B and JNK activation in TLR-mediated signaling pathways

Previous studies have reported that TAK1 plays a critical role in regulating the activation of multiple protein kinases during LPS signaling (Lee et al. 2000). We therefore examined the function of the TAK1 complex in TLR4-mediated signaling. Since all of the cells were weakly responsive to LPS, we cotransfected CD4/TLR4, a dominant active TLR4 chimera, together with either NF- κ B or AP-1-responsive luciferase reporter constructs. Using this system, wild-type, *Tab1*^{-/-}, and *Tab2*^{-/-} cells exhibit similar degrees of NF- κ B and AP-1 activation, while in *Tak1*^{m/m} cells, NF- κ B activation was partially decreased and AP-1 activation was markedly decreased (Fig. 3A). In contrast, IRF3 activation remains largely intact in wild-type, *Tak1*^{m/m}, *Tab1*^{-/-}, and *Tab2*^{-/-} cells. Therefore, these results indicate that in TLR4-mediated signaling, TAK1, but not TAB1 or TAB2, is required for AP-1 activation, but not for IRF3 activation. Intriguingly, our in vitro experiment showed that overexpression of kinase-inactive TAK1 mutant (TAK1-DN) inhibited IRF3 activation by CD4/TLR4 in a dose-dependent manner. This implies that overexpression of TAK1-DN may induce nonspecific inhibition of TLR4-induced IRF3 activation (Supplementary Fig. S1C,D). We next analyzed LPS-induced NF- κ B DNA-binding activity by using electrophoretic mobility gel-shift assay. As shown in Figure 3B, similar binding activities were observed in wild-type, *Tab1*^{-/-}, and *Tab2*^{-/-} cells in response to LPS. However, in contrast to TNF- α and IL-1 signaling, *Tak1*^{m/m} cells exhibited only a modest decrease in DNA-binding activity in response to LPS. To examine the function of the TAK1 complex in LPS-induced JNK activation, we

performed immunoblotting with anti-phospho-JNK1/2 antibody (Fig. 3C). JNK phosphorylation following LPS stimulation occurs normally in wild-type, *Tab1*^{-/-}, and *Tab2*^{-/-} cells, while it is severely impaired in *Tak1*^{m/m} cells. Therefore, these results suggest that while TAK1, but not TAB1 or TAB2, is required for JNK activation in a TLR4-mediated signaling pathway, it is only partially required for NF- κ B activation in response to LPS.

To assess whether this partial dependence on TAK1 in LPS signaling is due to the divergent nature of signaling downstream of TLR4—through both MyD88-dependent and independent pathways using the adapters TRIF and TRAM—we examined the role of TAK1 in signaling from a TLR that has a more linear path of activation to NF- κ B. Ligation of TLR3 by poly(I:C) leads to TAK1 activation through TRIF-TRAF6 (Sato et al. 2003), followed by NF- κ B activation (Jiang et al. 2003). However, TAK1 activation is not required for IRF3 activation through TLR3 (Jiang et al. 2004). We therefore analyzed TLR3-mediated NF- κ B and IRF3 activation using the luciferase-reporter assays (Fig. 3D). Wild-type, *Tab1*^{-/-}, and *Tab2*^{-/-} cells normally induced NF- κ B and IRF3 activation in response to poly(I:C); however, in *Tak1*^{m/m} cells, NF- κ B activation following poly(I:C) stimulation was significantly decreased, although IRF3 activation was largely intact. This is consistent with in vitro experiments that indicate overexpression of TAK1-DN inhibits poly(I:C)-induced NF- κ B activation in a dose-dependent manner, but not IRF3 activation (Supplementary Fig. S1E,F). Therefore, these results indicate that TAK1, but not TAB1 or TAB2 is required for TLR3-mediated NF- κ B activation, but not IRF3 activation. We next used immunoblotting analysis with antibodies specific for I κ B α and phospho-JNK1/2 to analyze poly(I:C)-induced NF- κ B and JNK activation, respectively (Fig. 3E). In wild-type, *Tab1*^{-/-}, and *Tab2*^{-/-} cells, I κ B α degradation occurs after 15 min post-induction with poly(I:C), however, degradation is reduced and significantly delayed in *Tak1*^{m/m} cells. Therefore, these results suggest that TAK1, but not TAB1 or TAB2 functions in early NF- κ B activation through TLR3. Surprisingly, *Tak1*^{m/m}, *Tab1*^{-/-}, and *Tab2*^{-/-} cells all exhibited delayed JNK phosphorylation in response to poly(I:C). Therefore, these results suggest that in TLR3-mediated signaling pathways, TAK1, but not TAB1 or TAB2, is required for NF- κ B

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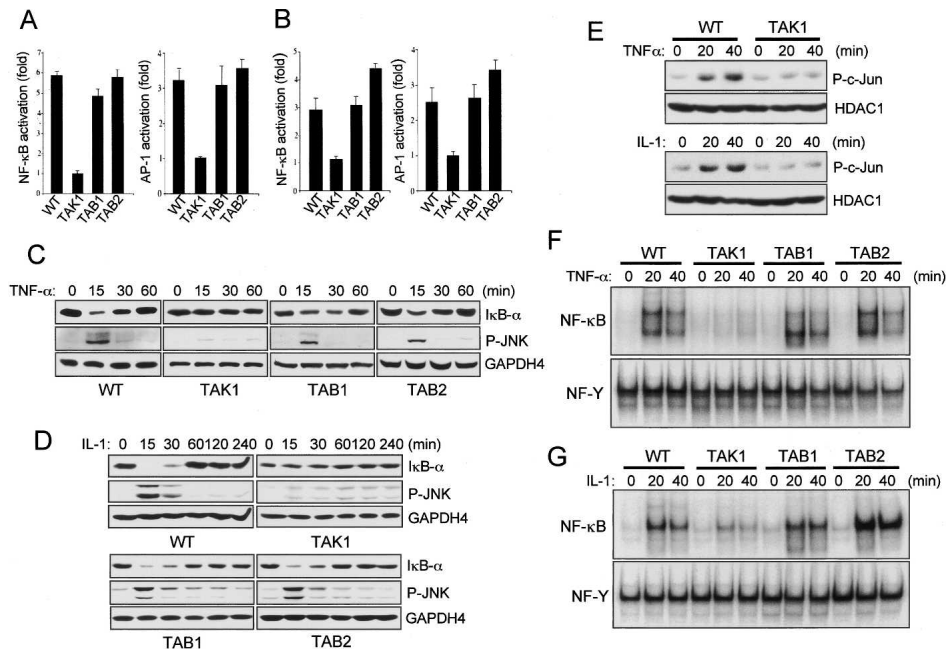


Figure 2. TAK1 deficiency inhibits NF- κ B and JNK activation by TNF- α and IL-1. (A,B) Wild-type (WT), *Tak1^{tm/m}* (TAK1), *Tab1^{-/-}* (TAB1), and *Tab2^{-/-}* (TAB2) cells were transfected with either pBIIX-luc (left) or AP1-luc (right) reporter together with *Renilla* luciferase vector. Twenty-four hours after transfection, cells were treated with TNF- α (10 ng/mL; A) or IL-1 (10 ng/mL; B) for 5 h and then analyzed for luciferase activity. Results are expressed as the fold induction in luciferase activity relative to that of untreated cells. Error bars indicate standard deviation. (C,D) I κ B- α degradation and JNK phosphorylation by TNF- α (C) or IL-1 (D) were analyzed by immunoblotting with antibodies specific for I κ B- α and phospho-JNK, respectively. Immunoblotting with anti-GAPDH4 antibody was performed as a loading control. (E) Cells were treated with TNF- α (10 ng/mL; top) or IL-1 (10 ng/mL; bottom) for the indicated time, nuclear extracts were prepared and immunoblotted with anti-phospho-c-Jun antibody. Immunoblotting with anti-HDAC1 antibody was performed as a loading control. (F,G) Cells were treated with TNF- α (10 ng/mL; E) or IL-1 (10 ng/mL; F) for the indicated times and nuclear extract were prepared. NF- κ B DNA-binding activity was analyzed by EMSA.

activation, but not for JNK activation and IRF3 activation. Taken together, TAK1 plays a differential role in signaling from TLR3 and TLR4 that probably reflects the complexity of the pathways that emanate from these receptors.

Tak1^{tm/m} cells are highly sensitive to TNF- α -induced apoptosis

Activation of NF- κ B following TNF- α stimulation protects cells from TNF- α -induced apoptosis (Beg and Baltimore 1996; Van Antwerp et al. 1996). In p65, IKK β and NEMO/IKK γ -deficient MEFs, defective NF- κ B activation increases their sensitivity to TNF- α -induced apoptosis (Beg et al. 1995; Tanaka et al. 1999; Rudolph et al. 2000). To examine whether loss of each member of the TAK1 complex affects TNF- α -induced apoptosis, we treated cells with TNF- α for 8 h, and analyzed cell death by using Annexin-V and PI staining (Fig. 4A). In agreement with our analysis of NF- κ B activation, TNF- α alone did not induce any apoptosis in wild-type, *Tab1^{-/-}*, and *Tab2^{-/-}* cells, whereas *Tak1^{tm/m}* cells were highly sensitive to TNF- α -induced apoptosis. Consistent with this result, immunoblotting analysis with anti-Caspase 8 antibody showed that TNF- α induced cleavage of pro-Caspase 8 (55 KD) to active Caspase-8 (43 KD) in

Tak1^{tm/m} cells, but not wild-type, *Tab1^{-/-}*, and *Tab2^{-/-}* cells (Fig. 4B). Therefore, these results indicate that TNF- α induces apoptosis in *Tak1^{tm/m}* cells through Caspase 8 activation. To examine the kinetics of TNF- α -induced apoptosis in *Tak1^{tm/m}* cells, we treated wild-type, *p65^{-/-}*, and *Tak1^{-/-}* cells with TNF- α and analyzed cell death by using Annexin-V and PI staining at multiple time points. As shown in Figure 4C, wild-type cells are resistant to TNF- α -induced apoptosis, whereas both *p65^{-/-}* and *Tak1^{-/-}* cells are highly sensitive to TNF- α -induced apoptosis. Intriguingly, *Tak1^{tm/m}* cells exhibited higher sensitivity to TNF- α -induced apoptosis than *p65^{-/-}* cells. Recent studies have reported that TAK1 is essential for regulation of IAP-mediated JNK1 activation, which plays a role in protecting cells from TNF- α -induced apoptosis (Sanna et al. 2002). In addition, the JNK/JunD pathway transduces a survival signal in collaboration with NF- κ B following TNF α stimulation. In the absence of NF- κ B activation, however, the JNK pathway mediates pro-apoptotic signaling in response to TNF α (Lamb et al. 2003). Therefore, our results suggest that TAK1 deletion increases sensitivity to TNF- α -induced apoptosis due to defects of both NF- κ B and JNK activation. We next tested whether exogenous expression of TAK1 proteins can rescue the mutant phenotypes of *Tak1^{tm/m}* cells (Fig. 4D). Cells were cotransfected with

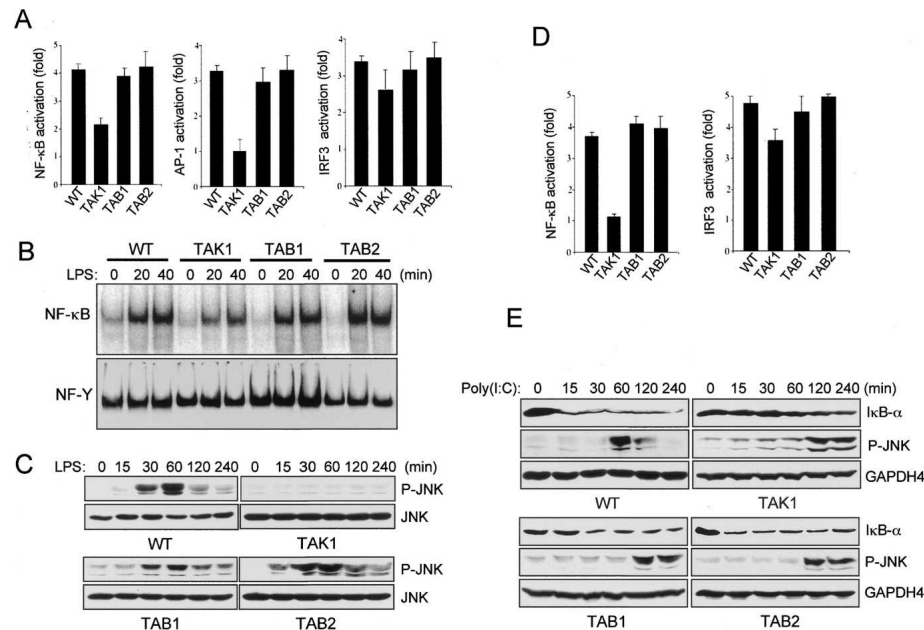


Figure 3. TLR-mediated NF- κ B and JNK activation is impaired in *Tak1^{tm/m}* cells. (A) Wild type, *Tak1^{tm/m}*, *Tab1^{-/-}*, and *Tab2^{-/-}* cells were cotransfected with CD4/TLR4 and *Renilla* luciferase vectors together with either pBIIx-luc (left), AP1-luc (middle), or IRF3-luc (right) reporters. Twenty-four hours after transfection, cells were lysed and analyzed for luciferase activity. Results are expressed as fold change in luciferase activity relative to untreated cells. (B) Cells were treated with LPS (1 μ g/mL) for the indicated times, nuclear extracts were prepared, and NF- κ B DNA-binding activity was analyzed by EMSA. (C) LPS-induced JNK phosphorylation was analyzed by immunoblotting with antiphospho-JNK antibody. Immunoblotting with anti-GAPDH4 antibody was performed to control for gel loading. (D) Cells were transfected with pBIIx-luc (left) or IRF3-luc (right) reporters together with *Renilla* luciferase vector. Twenty-four hours after transfection, cells were treated with poly(I:C) (5 μ g/mL) and luciferase activity was analyzed as in A. (E) Poly(I:C)-induced I- κ B- α degradation and JNK phosphorylation were analyzed by immunoblotting with anti-I- κ B- α and antiphospho-JNK antibodies, respectively. Immunoblotting with anti-GAPDH4 antibody was performed to control for gel loading.

GFP together with either pcDNA3 or wild-type TAK1 (TAK1-WT), and treated with TNF- α for 4 h. As expected, *Tak1^{tm/m}* cells expressing pcDNA3 undergo TNF- α -induced apoptosis; however, cells expressing exogenous TAK1 proteins are resistant to apoptosis. Thus, exogenous TAK1 expression restored resistance to TNF- α -induced apoptosis in *Tak1^{tm/m}* cells.

TAK1 functions upstream of the IKK complex and downstream of RIP1-TRAF2 and MyD88-IRAK1-TRAF6 in TNF α and IL-1/TLR signaling pathways

Activation of the IKK complex is essential for NF- κ B activation by various inflammatory cytokines (Hayden and Ghosh 2004). We therefore analyzed TNF- α and IL-1-induced IKK activation using in vitro kinase assays (Fig. 5A). In wild-type, *Tab1^{-/-}*, and *Tab2^{-/-}* cells, the IKK complex was activated normally with peak activity at 15 min post-induction of TNF- α , whereas IKK complex activation was severely impaired in *Tak1^{tm/m}* cells. In wild-type, *Tab1^{-/-}*, and *Tab2^{-/-}* cells, IL-1-induced IKK complex activation occurs normally and peaks at 30 min post-induction; however, IKK complex activation was moderately decreased in *Tak1^{tm/m}* cells. Consistent with these results, luciferase analysis with NF- κ B-re-

sponsive reporters showed that wild-type, *Tak1^{tm/m}*, *Tab1^{-/-}*, and *Tab2^{-/-}* cells all exhibited a similar degree of NF- κ B activation by overexpression of IKK β -SSEE, a dominant active mutant (Fig. 5B). Therefore, these results indicate that TAK1 functions upstream of the IKK complex in NF- κ B activation by TNF- α or IL-1, and that disruption of the *Tak1* gene does not affect signaling to NF- κ B downstream of the IKK complex. We next examined the requirement for TAK1 in phosphorylation of the IKK complex by TNF- α or IL-1 (Fig. 5C). Immunoblotting analysis with anti-phospho-IKK α/β antibody showed that phosphorylation of IKK α/β following TNF- α or IL-1 stimulation occurs normally and peaks at 15–30 min in wild-type cells, whereas in *Tak1^{tm/m}* cells, TNF- α -induced phosphorylation was significantly decreased and IL-1-induced phosphorylation was modestly decreased. Hence, these results suggest that TAK1 plays a differential role in regulating activation of the IKK complex in TNF- α and IL-1 signaling pathways. Taken together, TAK1 functions upstream of the IKK complex, where TAK1 mediates phosphorylation and activation of the IKK complex in TNF- α and IL-1 signaling pathways.

In the TNF- α signaling pathway, RIP1 and TRAF2 are required for IKK complex activation and recruitment of the IKK complex to TNFR1 receptor complex, respectively (Devin et al. 2000; Chen and Goeddel 2002). To

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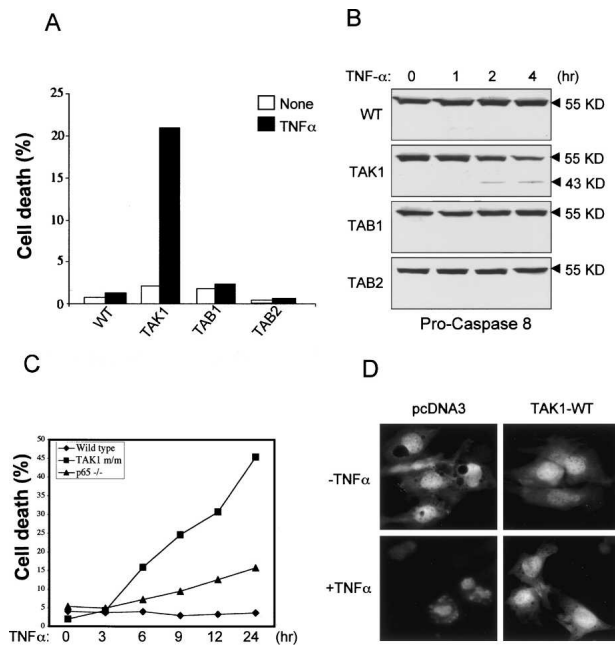


Figure 4. *Tak1^{tm/m}* cells are highly sensitive to TNF- α -induced apoptosis. (A) Wild-type (WT), *Tak1^{tm/m}*, *Tab1^{-/-}*, and *Tab2^{-/-}* cells were treated with TNF- α (10 ng/mL) for 8 h. Apoptosis was analyzed by staining with FITC-Annexin V and propidium iodide (PI) staining. Data given is the percentage of cells that stained positive with both PI and Annexin V. (B) Cells were stimulated with TNF- α for the indicated times, lysed, and immunoblotted with anti-caspase 8 antibody. Immunoblotting with anti-GAPDH4 antibody was performed to control for gel loading. (C) Wild-type, *p65^{-/-}* and *Tak1^{tm/m}* cells were treated with TNF- α (10 ng/mL) for the indicated times. Apoptosis was analyzed by FITC-Annexin V and propidium iodide (PI) staining as in A. (D) Cells were transfected with the GFP expression vector together with pcDNA3, or HA-tagged wild-type TAK1 (TAK1-WT). Twenty-four hours after transfection, cells were treated with TNF- α (10 ng/mL) for 4 h and analyzed by fluorescent microscopy.

test whether TAK1 is required for TRAF2 or RIP1-mediated IKK complex activation, wild-type and *Tak1^{tm/m}* cells were transfected with HA-tagged IKK β together with pcDNA3, Flag-tagged TRAF2, or RIP1 (Fig. 5D). In vitro kinase assays showed that wild-type cells exhibited IKK complex activation in the presence of TRAF2 or RIP1, whereas TRAF2 or RIP1-induced IKK complex activation was impaired in *Tak1^{tm/m}* cells. Thus, this result indicates that TAK1 is required for signaling from TRAF2 and RIP1 to the IKK complex. Given that MyD88, IRAK1/4, and TRAF6 are key regulators in IL-1R- and TLR signaling pathways (Takeda and Akira 2005), we next tested MyD88 or TRAF6-mediated activation of the IKK complex in wild-type and *Tak1^{tm/m}* cells (Fig. 5E). Cells were transfected with HA-tagged IKK β together with pcDNA3, Flag-tagged MyD88, or TRAF6, and the IKK complex activity was analyzed by in vitro kinase assay. As expected, expression of MyD88 or TRAF6 induced activation of the IKK complex in wild-type cells, whereas *Tak1^{tm/m}* cells exhibited an impaired

activation of the IKK complex. In addition, immunoblotting analysis with anti-IRAK1 antibody showed that IL-1-induced IRAK1 degradation occurs normally in both wild-type and *Tak1^{tm/m}* cells (Fig. 5F). Thus, these results indicate that in the IL-1 signaling pathway, TAK1 is required for signaling from MyD88 or TRAF6 to the IKK complex, but not for IRAK degradation. Therefore, these results suggest that TAK1 plays a role in mediating IKK complex activation from TRAF2-RIP1 and MyD88-TRAF6-IRAK1 in TNFR1 and IL-1R/TLR-mediated signaling pathways, respectively.

To examine whether exogenous expression of TAK1 proteins can restore IKK activity in *Tak1^{tm/m}* cells, wild-type and *Tak1^{tm/m}* cells were transfected with Flag-tagged IKK β together with pcDNA3 or HA-tagged wild-type TAK1 (Fig. 5G). In vitro kinase assays showed that *Tak1^{tm/m}* cells expressing exogenous TAK1 proteins exhibited IKK complex activation in response to TNF- α or IL-1, whereas IKK complex activation was impaired in the cells expressing pcDNA3. Thus, exogenous TAK1 expression restored activity of the IKK complex in TNF- α or IL-1 signaling pathways. Taken together, it appears that TAK1 functions upstream of IKK complex as an IKK kinase, and downstream of TRAF2-RIP1 and MyD88-TRAF6-IRAK1. Hence, TAK1 mediates IKK complex activation from the receptor complex, including TRAF2-RIP1 and MyD88-TRAF6-IRAK1 in TNF- α and IL-1-induced signaling pathways, respectively.

Since ERK and p38 MAP kinases are essential downstream molecules in TNFR1 and IL-1R/TLR-mediated signaling pathways (Baud and Karin 2001; Takeda and Akira 2005), we analyzed the kinetics of ERK and p38 MAP kinase activation by immunoblotting with antibodies specific for phospho-ERK1/2 and phospho-p38 MAP kinases (Supplementary Fig. S2A,B). Wild-type and *Tak1^{tm/m}* cells both exhibited phosphorylation of ERK and p38 MAP kinases in response to TNF- α , although phosphorylation of p38 MAP kinase is modestly decreased in *Tak1^{tm/m}* cells. In contrast, in *Tak1^{tm/m}* cells, ERK phosphorylation was delayed and p38 phosphorylation was severely impaired following IL-1 stimulation, relative to wild-type cells in which ERK and p38 phosphorylation occurs normally. Therefore, these results suggest that TAK1 plays a differential role in regulating activation of ERK and p38 MAP kinases in TNF- α and IL-1-induced signaling pathways.

TAK1 is not required for alternative NF- κ B activation in LT- β signaling pathway

In the alternative NF- κ B activation pathway, IKK α homodimers are activated via NF- κ B-inducing kinase (NIK) in response to cytokines such as LT- β , BAFF, and CD40 (Dejardin et al. 2002; Derudder et al. 2003). Activated IKK α , in turn, phosphorylates the NF- κ B p100 protein and induces its processing to the mature p52 subunit (Xiao et al. 2004). Finally, p52-containing heterodimers, notably p52:RelB, translocate into the nucleus and drive expression of a subset of NF- κ B target genes (Xiao et al.

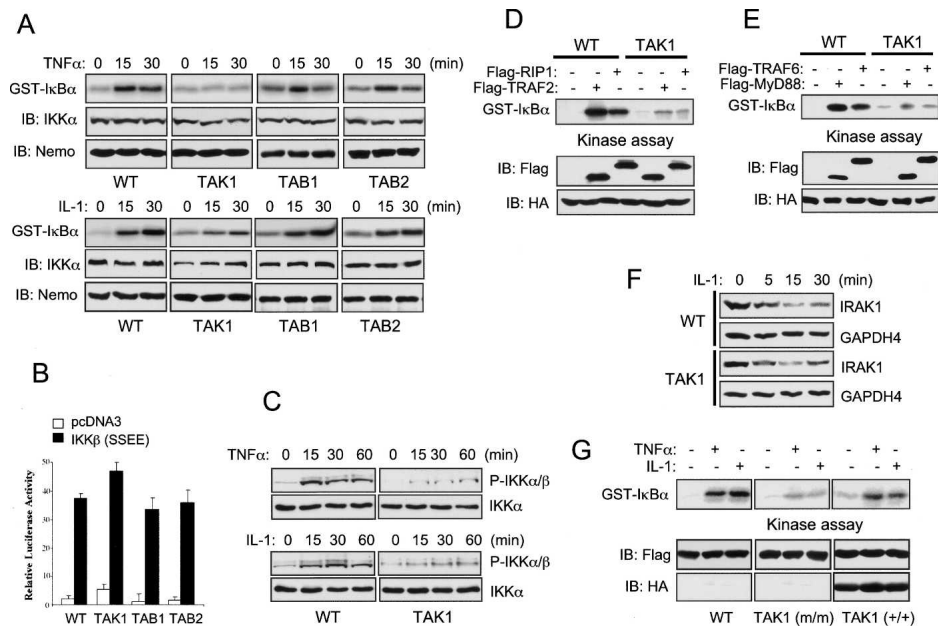


Figure 5. TAK1 is required for TNF- and IL-1-induced IKK activation. (A) Wild-type (WT), *Tak1^{mm}*, *Tab1^{-/-}*, and *Tab2^{-/-}* cells were treated with TNF- α (10 ng/mL; *top*) or IL-1 (10 ng/mL; *bottom*) for the indicated times. IKK activity was analyzed by in vitro kinase assay using a GST-I κ B α substrate. Protein input was analyzed by immunoblotting analysis with antibodies specific for IKK α and Nemo. (B) Cells were transfected with pcDNA3 or IKK β (SSEE) together with pBIIx-luc and *Renilla* luciferase vectors. NF- κ B activity was analyzed by luciferase assay and is expressed as fold change compared with untreated control. (C) Wild-type (WT) and *Tak1^{mm}* cells were treated with TNF- α (10 ng/mL; *top*) or IL-1 (10 ng/mL; *bottom*) for the indicated times, and then immunoblotted with antiphospho-IKK α / β antibody. Immunoblotting analysis with anti-IKK α antibody was performed as a control. (D) Cells were transfected with HA-IKK β together with either pcDNA3, Flag-tagged TRAF2, or RIP1 expression vector. Cells were harvested 24 h after transfection, and cells were immunoprecipitated with anti-HA antibody and protein A agarose. IKK β kinase activity was analyzed by in vitro kinase assay as in A. (E) Cells were transfected with HA-IKK β expression vector together with either pcDNA3, Flag-tagged TRAF6, or MyD88 expression vector. Cells were harvested 24 h after transfection and IKK β was immunoprecipitated with anti-HA antibody and protein A agarose. IKK β activity was analyzed by in vitro kinase assay. (F) Cells were treated with IL-1 (10 ng/mL) for the indicated times, and then immunoblotted with anti-IRAK1 antibody. Immunoblotting with anti-GAPDH4 antibody was performed to control for gel loading. (G) Wild-type and *Tak1^{mm}* cells were transfected with Flag-IKK β expression vector together with pcDNA3, or HA-tagged wild-type TAK1 (HA-TAK1-WT). Cells were stimulated 24 h later with TNF- α (10 ng/mL) or IL-1 (10 ng/mL) for 20 min, lysed, and immunoprecipitated IKK β kinase activity was analyzed by in vitro kinase assay.

2001; Bonizzi and Karin, 2004; Hayden and Ghosh 2004). To examine the role of TAK1 in the alternative NF- κ B pathway, we treated wild-type and *Tak1^{mm}* cells with TNF- α or LT- β , and analyzed NF- κ B-responsive gene expression using a luciferase-reporter assay (Fig. 6A). As expected, TNF- α -induced NF- κ B activation occurs normally in wild-type cells, but is completely abolished in *Tak1^{mm}* cells. In contrast, wild-type and *Tak1^{mm}* cells exhibited a similar degree of NF- κ B activation in response to LT- β , indicating that LT- β -induced, NF- κ B activation is normal in the absence of TAK1. NF- κ B activation by NIK overexpression was partially decreased in *Tak1^{mm}* cells, relative to wild-type cells (Fig. 6B), which is consistent with previous reports that NIK overexpression strongly induces both classical and alternative NF- κ B activation pathways (Malinin et al. 1997; Woronicz et al. 1997). These results, therefore, suggest that in the LT- β signaling pathway, TAK1 mediates signaling of NIK to the classical NF- κ B activation pathway, but is not involved in activation of the alternative NF- κ B pathway. To further test this hypothesis, we treated cells with LT- β at multiple time points and immunoblotted with the anti-p100 antibody (Fig. 6C). Wild-type and *Tak1^{mm}*

cells both exhibited a similar degree of induction of p100 processing to p52 subunit in response to LT- β , indicating that LT- β -induced p100 processing is normal in *Tak1^{mm}* cells. Therefore, TAK1 is not involved in activation of IKK α in the alternative NF- κ B pathway.

Role of TAK1 complex in TGF- β signaling pathway

TAK1, originally identified as a key regulator in TGF- β signaling, is believed to be involved in mesoderm induction and patterning through BMP signaling during *Xenopus* early development (Yamaguchi et al. 1995; Shibuya et al. 1998). To examine the role of each member of the TAK1 complex in TGF- β signaling, we analyzed TGF- β , NF- κ B, and AP-1-responsive gene induction in response to TGF- β signaling by using luciferase reporters (Fig. 7A). TGF- β -induced NF- κ B and AP-1 activation were impaired in the absence of TAK1, although NF- κ B and AP-1 activation following TGF- β stimulation occurs normally in wild-type, *Tab1^{-/-}*, and *Tab2^{-/-}* cells. Consistent with these results, JNK phosphorylation was completely abolished in *Tak1^{mm}* cells relative to wild-type, *Tab1^{-/-}*,

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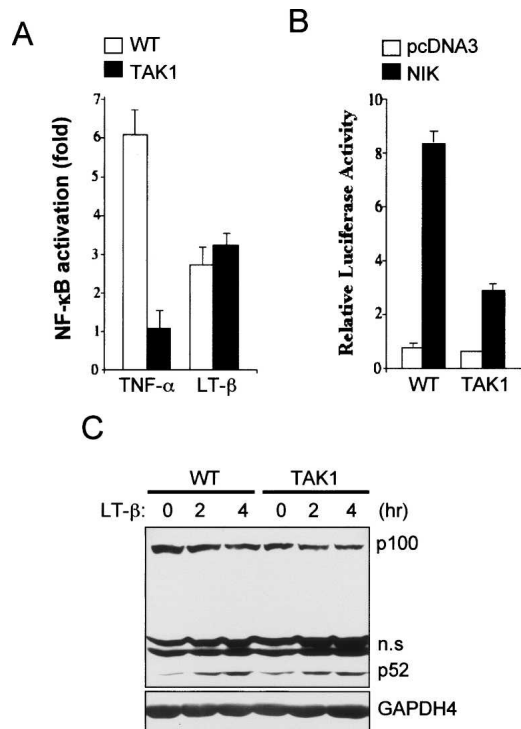


Figure 6. LT- β -induced NF- κ B activation is normal in *Tak1^{tm/m}* cells. (A) Wild-type (WT) and *Tak1^{tm/m}* cells were transfected with pBIIx-luc reporter along with *Renilla* luciferase vector. Twenty-four hours after transfection, cells were treated with TNF- α (10 ng/mL) or anti-LT- β R antibody (2 μ g/mL) for 5 or 12 h, respectively, and then analyzed for luciferase activity. Results are expressed as the fold change in luciferase activity relative to untreated cells. (B) Cells were transfected with pcDNA3 or NIK expression vector along with pBIIx-luc reporter and *Renilla* luciferase vector. NF- κ B activity was analyzed by luciferase assay. (C) Cells were stimulated with anti-LT- β R antibody (2 μ g/mL) for the indicated times, and then immunoblotted with anti-p100 antibody. Protein amount was analyzed by immunoblotting with anti-GAPDH4 antibody.

and *Tab2^{-/-}* cells in which the phosphorylation occurs normally (Fig. 7B). Intriguingly, wild-type, *Tak1^{tm/m}*, and *Tab2^{-/-}* cells all exhibit normal gene expression in response to TGF- β , whereas gene expression was severely impaired in *Tab1^{-/-}* cells. Thus, these results indicate that in TGF- β -induced signaling pathways, TAK1 is required for NF- κ B and JNK activation, but not for gene induction, TAB1 is required for gene induction but not for NF- κ B and JNK activation, and TAB2 is not required for gene induction or activation of NF- κ B and JNK. This implies that TAK1 and TAB1, but not TAB2 play differential roles in TGF β signaling pathways. We next examined TGF β -induced Smad2 activation by immunoblotting and immunostaining with antiphospho-Smad2 antibody (Fig. 7B,C). Loss of TAK1, TAB1, or TAB2 does not affect phosphorylation or nuclear translocation of Smad2 following TGF- β stimulation. This indicates that the TAK1 complex is not required for TGF- β -induced Smad2 activation—the classical TGF- β signaling pathway. Taken

together, these results suggest that each member of the TAK1 complex plays different roles in TGF- β signaling pathways. TAB1 is required for efficient induction of a TGF β -responsive reporters, TAK1 is required for TGF β -induced NF- κ B, and AP-1 activation and deficiency in TAB2 does not appear to affect TGF β signaling.

Discussion

The inducible transcription factors AP-1 and NF- κ B are key mediators of multiple aspects of the immune and inflammatory response and are activated upon stimulation with proinflammatory cytokines, microbial products, and various cell stressors. Multiple receptors, most notably the Toll/IL-1 and TNF receptor families, have

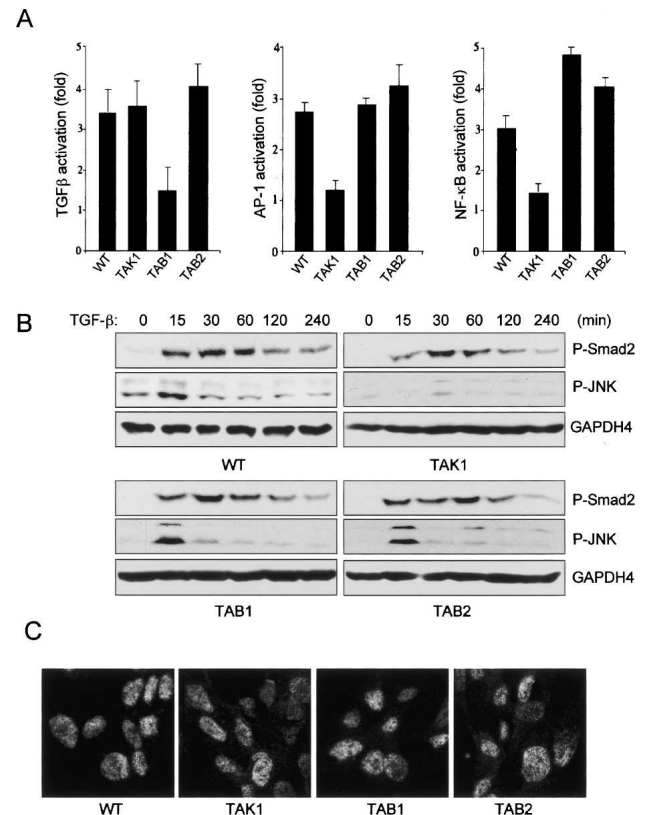


Figure 7. Role of TAK1 complex in TGF- β signaling pathway. (A) Wild-type, *Tak1^{tm/m}*, *Tab1^{-/-}*, and *Tab2^{-/-}* cells were transfected with the either 3TP-luc (left), AP-1-luc (middle), or pBIIx-luc (right) reporter construct together with *Renilla* luciferase vector. Twenty-four hours after transfection, cells were treated with TGF- β (1 ng/mL) for 24 h and then analyzed for luciferase activity. Results are expressed as the fold induction in luciferase activity relative to that of untreated cells. (B) Smad2 and JNK phosphorylation by TGF- β (1 ng/mL) were analyzed by immunoblotting with antiphospho-Smad2 antibody and antiphospho-JNK antibody, respectively. Immunoblotting analysis with anti-GAPDH4 antibody was performed as a control. (C) Cells were stimulated with TGF- β (1 ng/mL) for 20 min, stained with antiphospho-Smad2 antibody, and analyzed by immunofluorescence microscopy.

been shown to activate both AP-1 and NF- κ B, and activation of the IKK complex and JNK have been considered the points of convergence in signaling pathways leading to NF- κ B and AP-1. Recently, however, the protein kinase TAK1 has emerged as a potential regulator of both these pathways, acting upstream of IKK and JNK as both an IKK kinase (IKKK) and JNK kinase kinase (JNKKK). Initial support for this hypothesis came from a variety of in vitro experiments that suggested that the interaction of the TAK1 complex (TAK1, TAB1, and TAB2) with ubiquitinated TRAF family members led to TAK1 activation, followed by activation of IKK and JNK. Although the biochemical evidence for such a role for the TAK1 complex appeared persuasive, there is almost no definitive genetic evidence supporting the hypothesis that TAK1, TAB1, and TAB2 are activated through a ubiquitination-dependent mechanism, and that the TAK1 complex plays such an essential role in AP-1 and NF- κ B signaling pathways in mammalian cells.

Therefore, to investigate the biological role of TAK1, we generated TAK1-deficient mice by using gene trap mutated *Tak1* ES (Fig. 1A; Stryke et al. 2003; Austin et al. 2004; Skarnes et al. 2004). *Tak1* homozygous mutant embryos died around E10 and exhibited abnormal development of the neural tube, suggesting that TAK1 is critical for early embryonic development (Fig. 1D,E). The phenotype of *Tak1* embryos is strikingly different from that of *Tab1* and *Tab2*-deficient embryos, since *Tab1* mutant embryos die at a late stage of gestation due to abnormal cardiovascular and lung morphogenesis, while *Tab2* mutant embryos die around E12.5 due to liver degeneration and apoptosis (Komatsu et al. 2002; Sanjo et al. 2003). These results further emphasize that TAK1 has one or more unique functions that are independent of the adaptor proteins, TAB1 and TAB2.

In the NF- κ B activation pathway, TAK1 interacts with TRAF2 or TRAF6, and IKK α / β following TNF- α or IL-1 stimulation, and reduction of TAK1 through RNA interference inhibits IL-1 and TNF- α -induced IKK activation (Takaesu et al. 2003). In the JNK activation pathway, TAK1 interacts directly with JNK1, and expression of a kinase-inactive TAK1 inhibits TNF- α -induced JNK activation (Sanna et al. 2002). Although primary *Tak1*^{m/m} embryonic fibroblasts could not be cultured, we were able to generate SV40 large T antigen-transformed embryonic fibroblasts for analyzing signaling in the absence of TAK1 (Kobayashi et al. 2005). We have found that loss of TAK1 leads to defects in NF- κ B and JNK-responsive reporter gene expression, NF- κ B DNA-binding activity, I κ B α degradation, IKK complex activation, and JNK phosphorylation in TNF- α signaling pathways. In contrast, NF- κ B and JNK activation following TNF- α stimulation was largely intact in *Tab1*^{-/-} and *Tab2*^{-/-} cells. Thus, these results indicate that TAK1, but not TAB1 or TAB2, is required for NF- κ B and JNK activation in TNF- α signaling pathways. In addition, in vitro kinase assays in *Tak1*^{m/m} cells demonstrate that TAK1 is necessary for signaling from RIP1 and TRAF2 to the IKK complex (Fig. 5). Thus, TAK1 is a key functional intermediate between the recruitment of TRAF2-RIP1 to

TNFR1 (Devin et al. 2000; Chen and Goeddel 2002) and activation of the IKK complex. It is well established that NF- κ B and JNK activation plays a role in protecting cells from TNF- α -induced apoptosis (Beg and Baltimore 1996; Van Antwerp et al. 1996; Lamb et al. 2003). Consequently, *Tak1*^{m/m} cells were highly sensitive to apoptosis and display elevated levels of caspase 8 activation relative to wild-type, *Tab1*^{-/-}, and *Tab2*^{-/-} cells (Fig. 4). As expected, exogenous TAK1 expression could rescue the defect of *Tak1*^{m/m} cells in TNF- α -induced apoptosis. In contrast to previous in vitro studies that have suggested that the TAK1 adaptor proteins TAB1 and TAB2 are critical for TAK1 activation and TNF- α signaling, our results suggest that only TAK1 is required for TNF- α -induced IKK activation and JNK phosphorylation leading to NF- κ B and JNK activation in vivo.

In vitro studies have reported that the MyD88-IRAK1-IRAK4-TRAF6 complex regulates TAK1 activation through TAB1 and TAB2, thereby leading to IKK complex and MKK6 activation in response to IL-1 (Wang et al. 2001; Jiang et al. 2002). However, when we examined the role of TAK1, TAB1, and TAB2 in the IL-1 signaling pathway, only TAK1 deficiency abolished IL-1-induced NF- κ B and JNK activation. Intriguingly, NF- κ B DNA-binding activity and IKK complex activity following IL-1 stimulation were not completely inhibited in *Tak1*^{m/m} cells, although no NF- κ B-responsive reporter gene expression was observed following TNF- α and IL-1 stimulation. One likely explanation for this discrepancy is that the poor transfection efficiency of embryonic fibroblasts precludes using luciferase analysis to discriminate between the complete loss following TNF α and near-complete loss of NF- κ B activation following IL-1 that is detected by biochemical analyses. In vivo, these results suggest that although TAK1 is crucial for both IL-1 and TNF- α -induced JNK activation, the requirement for TAK1 in IL-1-induced IKK complex and NF- κ B activation is less complete. In vitro kinase assays indicate that the requirement for TAK1 function is positioned between IRAK-MyD88-TRAF6 and activation of the IKK complex (Fig. 5). Considered together with the result that TAK1 deficiency caused delayed ERK activation and impaired p38 activation in response to IL-1 (Supplementary Fig. S2), TAK1 may function as a branching signal transducer from the MyD88-IRAK1-TRAF6 complex to the NF- κ B, JNK, ERK, and p38 MAP kinase pathways (Fig. 8).

Previous studies have demonstrated that TAK1 is required for IKK complex and JNK activation in LPS-induced signaling (Lee et al. 2000). However, we find that while TAK1 deletion abolishes JNK-responsive reporter gene expression, NF- κ B DNA-binding affinity and reporter gene expression are only partially reduced (Fig. 3A-C). Alternatively, when TAK1 activity is disrupted by DN-TAK1 overexpression, consistent with previously published results (Lee et al. 2000), we find that NF- κ B activation by TLR4 is completely abrogated (Supplementary Fig. S1C). NF- κ B and JNK activation following LPS stimulation is normal in *Tab1*^{-/-} and *Tab2*^{-/-} cells; thus TAK1, but not TAB1 or TAB2, is required for LPS-in-

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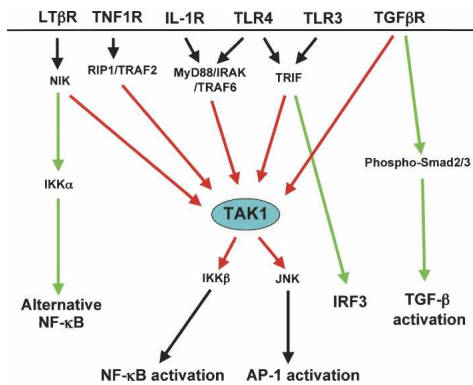


Figure 8. Schematic model showing role of TAK1 complex in inflammatory signaling pathways. Red lines indicate TAK1-dependent signaling pathways, while green lines indicate TAK1-independent pathways.

duced JNK activation. These results imply that TAK1 mediates signaling of MyD88–IRAK–TRAF6 complex to NF- κ B activation, while TRAM-mediated NF- κ B activation is altogether independent of TAK1. Previous observations that TAK1 is involved in all TLR4 signaling pathways to NF- κ B likely resulted from nonphysiological effects induced by overexpression of DN-TAK1 constructs. Consistent with this hypothesis, while *Tak1^{tm/m}* cells exhibit normal LPS-induced IRF3 activation, which is dependent on TRIF, overexpression of DN-TAK1 completely inhibits both NF- κ B and IRF3 activation. Our analysis of TLR3-induced NF- κ B activation further supports a selective role for TAK1 in TLR signaling. Following poly(I:C) stimulation, TAK1, but not TAB1 or TAB2, is required for NF- κ B activation, but not for IRF3 activation. Thus TAK1, but not the adapter proteins TAB1 and TAB2, appears to play a vital role in inflammatory signaling to both AP-1 and the classical NF- κ B pathway.

In recent years, two NF- κ B activation pathways have been recognized that play distinct roles in the regulation of immune responses in mammals (Bonizzi and Karin 2004; Hayden and Ghosh 2004). In the classical pathway, diverse stimuli, such as proinflammatory cytokines TNF- α and IL-1, pathogen-associated molecular patterns (PAMPs), and antigen receptors (i.e., TcR and BcR) induce phosphorylation and degradation of I κ Bs in an IKK β and NEMO-dependent manner. The so-called alternative pathway is instead dependent on IKK α . Cytokines, such as LT- β , BAFF, and CD40 activate IKK α via the protein kinase NIK, and the activated IKK α in turn induces processing of NF- κ B2/p100 precursor to mature p52, followed by translocation of p52 containing complexes to the nucleus (Senftleben et al. 2001). We found that TAK1 deletion does not affect NF- κ B reporter gene expression or the processing of p100 to p52 following LT- β stimulation. Interestingly, NF- κ B reporter gene expression following NIK overexpression, which activates both IKK α and IKK β (Woronicz et al. 1997), was moderately decreased in *Tak1^{tm/m}* cells. These results raise the intriguing possibility that TAK1 may be necessary for the IKK β

activation that occurs upon NIK overexpression, but that TAK1 is not required for NIK-mediated IKK α activation and p100 processing in LT- β signaling. Furthermore, these results highlight a distinct requirement for the activation of alternative and classical NF- κ B, upstream of the IKK complex itself, and suggest that TAK1 and NIK may occupy analogous positions as IKKs in these two signaling pathways.

It has been reported that TAK1 is involved in TGF- β -induced signaling of *Xenopus* and mammalian cells (Shibuya et al. 1996; Yamaguchi et al. 1995). To test requirements for each member of the TAK1 complex in TGF- β signaling, we analyzed luciferase activities by using NF- κ B, JNK, and TGF- β -responsive reporters. Intriguingly, TAB1 is required for efficient induction of TGF- β -responsive reporters, while TAK1 is required for TGF- β -induced NF- κ B and AP-1 activation, and deficiency in TAB2 does not appear to affect TGF- β signaling. However, the TAK1 complex, including TAK1, TAB1, and TAB2 is not required for Smad2 activation in TGF- β signaling. Therefore, these results suggest that each member of the TAK1 complex plays different roles in regulating NF- κ B, JNK, and Smad2 activation in TGF- β signaling pathways (Fig. 8). Whether the whole TAK1 complex is required for TGF- β -induced signaling remains to be determined by generating cells lacking TAK1 together with TAB1 and TAB2.

A surprising conclusion from our studies is the lack of any effect on TNF α , IL-1, LPS, and poly(I:C) signaling in cells lacking TAB1 or TAB2. At present, the early lethality of the individual knock-outs has prevented analysis of signaling in cells lacking both TAB1 and TAB2. In addition, the recent description of human TAB3 as a TAB2 homolog has further complicated the genetic analysis of the role of TAB proteins. The murine TAB3 gene has not yet been cloned, although efforts are underway to knock-out this gene. A recent study has reported that down-regulation of both TAB2 and TAB3 using RNA interference was necessary to observe inhibition of IKK and JNK activation by TNF- α and IL-1, suggesting that TAB2 and TAB3 may play a redundant role in these signaling pathways (Cheung et al. 2003; Ishitani et al. 2003). Therefore, it is clear that additional analysis will be necessary to determine the exact role that TAB proteins play in regulation of TAK1 activity. However, the different developmental phenotypes observed in TAK1, TAB1, and TAB2 knock-outs strongly suggest that the three proteins do not always function together as a complex. Remarkably, only the lack of TAB1 leads to a dramatic reduction of TGF- β -induced gene-expression, once again strongly hinting at a unique role for TAB1 that does not depend on either TAK1 or the other TAB-proteins. Our results also raise questions about the widely discussed model of ubiquitination-dependent activation of TAK1, where TAB1 is believed to play an essential role. While we have been unable to genetically address the role of ubiquitination in TAK1 regulation, it does appear that additional studies will be necessary to fully elucidate the details of how the different TAB proteins might regulate TAK1.

Materials and methods

Cells and antibodies

HEK 293 (human kidney embryonic cells) and COS1 (simian fibroblast cells) were purchased from ATCC. HEK 293, COS1, and immortalized MEF cells were cultured in DMEM medium (GIBCO) containing 7% FBS, 2 mM L-glutamine, 100 unit/mL of penicillin, and 100 µg/mL streptomycin and 5×10^{-5} M β -mercaptoethanol. Antibodies used were anti-TAK1 (Ninomiya-Tsuji et al. 1999), antiphospho-IKK α/β , antiphospho-c-Jun, antiphospho-Erk1/2, antiphospho-JNK1/2, anti-JNK1/2, antiphospho-p38, and antiphospho-Smad2 (Cell Signaling), anti-I κ B α (Santa Cruz, C21), and anti-GAPDH4 (Research Diagnostics). Cells were treated with poly(I:C) and LPS (Sigma), anti-LT- β R (Alexis Biochemicals), and rhTNF- α , mTGF- β , and mL-1 β (R&D Systems) as indicated.

Generation of Tak1^{m/m} mice

Tak1 gene trap ES clone (XB444) was obtained from Dr. Williams Skarnes (BayGenomics) (Stryke et al. 2003; Skarnes et al. 2004). The *Tak1* ES clones were injected into C57BL/6 blastocysts and transferred to ICR females. Male chimeric mice were mated with C57BL/6 females, resulting in transmission of the inserted allele to the germline. Positive mice were interbred and maintained on a mixed 129 \times C57BL/6 background.

Generation of MEFs

E9.75 embryos from timed breeding of *Tak1*^{-/-} mice were dissected free of maternal tissues and Reichert's membrane, washed with PBS, and incubated with 0.1% collagenase (Sigma) and Trypsin-EDTA (GIBCO) for 30 min at 37°C. The cell suspension was plated in 12-well plates containing DMEM supplemented with 10% fetal bovine serum. *Tak1*^{m/m} MEFs were identified by immunoblotting with TAK1 antibody or PCR using extraembryonic tissues. MEFs were stably transfected with linearized SV40 Large T antigen plasmid by using FuGENE 6.

Immunofluorescence

For immunofluorescence analysis, cells were grown on Alcian Blue-coated coverslips, treated with TGF- β (1 ng/mL) for 20 min, washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, and permeabilized with permeabilization buffer (0.05% saponin, 1% FBS, 10 mM HEPES, 10 mM glycine in PBS at pH 7.5) for 30 min at room temperature. Cells were incubated with antiphospho-Smad2 antibody for 30 min at room temperature, followed by incubation with goat anti-rabbit-FITC under identical conditions.

TNF-induced apoptosis

MEF cells (3×10^5) were seeded in a 6-cm Petri dish 24 h prior to TNF- α stimulation at the indicated concentration for the time. Cells were harvested in PBS and incubated with Annexin V-FITC and propidium iodide (BD Pharmingen) according to manufacturer's instructions and analyzed by flow cytometer.

Luciferase reporter assay

MEF cells (2.5×10^5) grown on 12-well plates were transiently transfected using FuGENE 6 with the NF- κ B-dependent reporter construct pBIIx-luc together with the *Renilla* luciferase vector

(Promega). Total DNA concentration in each experiment was maintained by adding the appropriate empty vector to the DNA mixture. Twenty-four hours after transfection, cells were lysed, and luciferase activity was measured using the dual luciferase assay kit (Promega). The levels of transfected proteins in lysates were examined by immunoblotting with appropriate epitope tag-specific antibodies.

In vitro kinase assay

For in vitro kinases assays, 1×10^7 MEF cells grown on a 10-cm Petri dish were transiently transfected with various DNAs using the FuGENE 6 reagent as described above. Forty-eight hours after transfection, the cells were lysed in TNT lysis buffer for 30 min. Alternatively, the cells were either untreated or treated with TNF (10 ng/mL) and IL-1 (10 ng/mL) and then lysed in TNT lysis buffer. Protein amount in each lysate was determined by using a Bio-Rad protein assay kit (Bio-Rad) and then normalized among the samples. Protein from lysates was immunoprecipitated using either anti-Nemo antibody and protein G-Sepharose or anti-Flag (M2)-coupled agarose beads overnight at 4°C, and the precipitates were washed in TNT lysis buffer and then kinase buffer (20 mM HEPES at pH 7.5, 20 mM MgCl₂, 1 mM EDTA, 2 mM NaF, 2 mM-glycerophosphate, 1 mM dithiothreitol, 10 µM ATP). Precipitates were incubated for 15 min at 37°C in kinase buffer containing appropriate GST-fused substrate proteins and 10 µCi of [γ ³²P]ATP (Amersham Biosciences). The substrate was then precipitated using glutathione-agarose (Amersham Biosciences), resolved by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography.

Galactosidase staining of embryos

β -Galactosidase staining was performed as previously described (Lawson et al. 1999).

Acknowledgments

We thank Williams Skarnes (BayGenomics) for providing *Tak1*^{m/m} ES cells. M.S.H. was supported by NIH/National Institute of General Medical Sciences Medical Scientist Training Grant GM07205. This work was supported by a grant from NIH (R37-AI33443).

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Genes Dev. 2005, **19**:

Access the most recent version at doi:[10.1101/gad.1360605](https://doi.org/10.1101/gad.1360605)

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