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Lys⁴⁸-linked TAK1 polyubiquitination at lysine-72 downregulates TNF α -induced NF- κ B activation via mediating TAK1 degradation

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Abstract

Protein kinases are important regulators of intracellular signal transduction pathways and play critical roles in diverse cellular processes. TAK1, a member of the MAPKKK family, is essential for TNF α -induced NF- κ B activation. Phosphorylation and Lys⁶³-linked polyubiquitination (polyUb) of TAK1 are critical for its activation. However, whether TAK1 is regulated by polyubiquitination-mediated protein degradation after its activation remains unknown. Here we report that TNF α induces TAK1 Lys⁴⁸ linked polyubiquitination and degradation at the later time course. Furthermore, we provide direct evidence that TAK1 is modified by Lys⁴⁸-linked polyubiquitination at lysine-72 by mass spectrometry. A K72R point mutation on TAK1 abolishes TAK1 Lys⁴⁸-linked polyubiquitination and enhances TAK1/TAB1 co-overexpression-induced NF- κ B activation. As expected, TAK1 K72R mutation inhibits TNF α -induced Lys⁴⁸-linked TAK1 polyubiquitination and degradation. TAK1 K72R mutant prolongs TNF α -induced NF- κ B activation and enhances TNF α -induced IL-6 gene expression. Our findings demonstrate that TNF α induces Lys⁴⁸-linked polyubiquitination of TAK1 at lysine-72 and this polyubiquitination-mediated TAK1 degradation plays a critical role in the downregulation of TNF α -induced NF- κ B activation.

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Keywords

TNF α ; TAK1; Lys⁴⁸-linked polyubiquitination; degradation

1. Introduction

Protein kinases are important regulators of intracellular signal transduction pathways and play critical roles in a plethora of cellular processes such as cell cycle regulation, apoptosis and immune response [1-3]. The human genome encodes over 500 protein kinase genes which constitute approximately 2% of human genes [4]. Perturbation of protein kinases can cause many types of human cancers [5]. Once a kinase is activated, its activity will be subsequently downregulated through negative feedback systems to attenuate or terminate kinase activity and the signaling pathway that it regulates. Several mechanisms, including endocytosis, reduction of the kinase mRNA, dephosphorylation and ubiquitin-proteasome system, are involved in the negative regulation of kinase mediated signal transduction pathway [6-12].

Tumor necrosis factor α (TNF α) is a proinflammatory cytokine that plays a critical role in innate immune response by eliciting inflammation [13]. Stimulation of cells with TNF α activates intracellular signaling pathways which lead to the activation of transcription factors such as NF- κ B [14]. Upon binding to its receptor, TNF α induces the formation of a receptor-associated complex composed by several adaptor proteins (TRADD, TRAF2, and TRAF5) and RIP1, which promotes Lys⁶³-linked polyubiquitination and subsequent activation of RIP1 and TRAF2 [15-17]. The activated RIP1 and TRAF2 lead to TAK1 Lys⁶³-linked polyubiquitination which is vital for its activation and signal transduction [18-22]. However, genetic evidence suggests that Tak1 but not Ripk1 is essential for TNF α -induced NF- κ B activation in mouse embryonic fibroblasts (MEFs) [23-25]. Upon Lys⁶³-linked polyubiquitination and activation, TAK1 causes activation of I κ B kinases (IKKs) to activate transcriptional factor NF- κ B [26].

Ubiquitin proteasome system plays a pivotal role in the regulation of NF- κ B pathway [9, 27-29]. For instance, after RIP1 activation, A20 ubiquitin editing complex targets RIP1 for inactivation by promoting Lys⁴⁸-linked polyubiquitination and degradation of RIP1 [30, 31]. Upon IKK β activation, Ro52 catalyzes the monoubiquitination of IKK β to down-regulate NF- κ B signaling while at resting stage the IKK β protein level is tightly regulated by another E3 ligase KEAP1 which promotes IKK β proteolysis [32-34]. TAK1 is an essential signaling mediator that bridges TNFR1 to the IKKs complex. Whether TAK1 is also under the surveillance of ubiquitin proteasome system and more importantly, exactly how it is regulated by such a system remain to be elucidated. Here, we report that TNF α induces Lys⁴⁸-linked polyubiquitination of TAK1 at lysine-72 and this polyubiquitination-mediated TAK1 degradation plays an important role in the downregulation of TNF α -induced NF- κ B activation. Collectively, our data reveal a new regulatory mechanism for inactivation of TNF α -induced NF- κ B activation.

2. Experimental procedures

2.1 Cell culture, antibodies and reagents

293T cells and *Tak1*-deficient mouse embryonic fibroblast (MEF) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in 5% CO₂. The following antibodies and reagents were used: TAK1 (4505), phospho-IKK α / β (2681), IKK β (2684) phospho-JNK (9251), JNK (9252L), phospho-I κ B α (9246L), I κ B α (9242) and

secondary antibodies conjugated to horseradish peroxidase (Cell Signaling); HA (sc-7392), RelA (sc-8008), Ubiquitin (sc-8017), PCNA (sc-56), and Protein A-agarose beads (Santa Cruz Biotechnology); FLAG-M2 (F3165) and β -actin (A2228) (Sigma); Lys⁶³-specific Ubiquitin (05-1308 and 05-1313) and Lys⁴⁸-specific Ubiquitin (05-1307) (Millipore); Recombinant mTNF α (R & D Systems); MG132 (Calbiochem).

2.2 Plasmids and transfection

Human TAK1 cDNA was inserted into the expression vector pcDNA3.1 with a Flag tag at its N-terminal. Site-directed mutagenesis was used to generate K72R point mutation. Retroviral TAK1 wild-type and K72R mutant constructs were generated using pBabe-puro vector. The NF- κ B-dependent *firefly* luciferase reporter and pCMV promoter-dependent *Renilla* luciferase reporter plasmids were purchased from Clontech. HEK-293T cells and TAK1-deficient MEF cells were transfected with expression plasmids using FuGene 6 (Roche) and Lipofectamine 2000 (Invitrogen), respectively.

2.3 Establishment of TAK1 stable MEF cell lines

Topo-Vector, Topo-Flag-TAK1 wild-type and Topo-FLAG-TAK1 K72R mutant were transfected into Tak1-deficient MEF cells. After 48 hours, transfected cells were selected by G418 (4 mg/ml) for 10 days. For retroviral infection, the pBabe empty vector, pBabe-TAK1 wild-type, pBabe-TAK1 K72R mutant were co-transfected with retrovirus packing vector PEGPAM 3e and PLC-ECO in HEK-293T cells to obtain retroviral supernatants. Viral supernatants were collected after 48 and 72 hours. TAK1-deficient MEF cells were incubated with retroviral supernatant in the presence of 4 μ g/ml polybrene. Stable cell lines were established after 10 days of puromycin (3 μ g/ml) selection.

2.4 Immunoprecipitation and immunoblotting

Cells were washed 3 times with ice-cold PBS, then lysated on ice with lysis buffer (25 mM HEPES (pH 7.7), 135 mM NaCl, 3 mM EDTA, 1% Triton X-100, 25 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM Benzamidine, 20 mM disodium p-nitrophenylphosphate, phosphatase inhibitor cocktail A and B from Sigma) for 30 min. After lysis, the whole protein extracts were collected by high-speed centrifuge at 15,000 g for 15 min at 4°C. For immunoprecipitation, primary antibodies were added to the supernatant and incubated with rotation for 3 h at 4°C. After brief centrifugation at 15,000 g for 5 min, the supernatant was carefully transferred to a new tube and incubated with protein A-agarose beads for 2 hours at 4°C. After binding, beads were washed 3 times with ice-cold lysis buffer and boiled in the Laemmli buffer for 10 min. The immunoprecipitates were resolved on a SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

2.5 Immunoprecipitation and mass spectrometry

Five dishes (10 cm in diameter) of 293T cells were transiently transfected with Flag-TAK1 expression vector. Cells were lysed in NETN (20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 0.5 mM EDTA, 0.5 % (v/v) Nonidet P-40 (NP-40)) buffer containing 1% SDS (Sodium Dodecyl Sulfate), and then boiled at 95 degree for 10 minutes to dissociate the interaction proteins. After boiling, the lysis was diluted 10 fold in NETN, centrifuged at 15,000 rpm and incubated with anti-FLAG antibody to immunoprecipitate Flag-TAK1. The immunoprecipitates were then eluted and boiled in SDS Laemmli buffer, resolved on a SDS PAGE and gel regions above 70 kDa were cut and in-gel digested by trypsin as previously described [9, 27, 28]. Purified peptides were analyzed by a Linear Tandem Quadrupole (LTQ) mass spectrometer (Thermo Fisher) equipped with a nanospray ESI source and an

online HPLC system coupled to an auto sampler. The purified peptides were injected in a self-packed C18 column (5cm bedding, 75 μ m inner diameter and 5 μ m particle size) and eluted with 55-min gradient of 95% solvent A (0.1% formic acid in water) to 30% solvent B (0.1% formic acid in acetonitrile) with a flow rate of ~450nl/min. A data-dependent experiment was carried out where the top 20 most intensive ions were triggered by collision induced disassociation (CID) for MS2. The precursor isolation window was set as 3 units. Raw data were searched for lysine ubiquitination against a human ref database (2009.06.27) by Sequest embedded in the Bioworks Browser (Thermo Fisher). Key parameters used for the search are: precursor and fragment mass tolerances were confined within 4.0 Da and 0.5 Da, respectively and a maximum of two missed cleavage sites were allowed. Other parameters for search include: a fixed cysteine-destreak (+75 Da), and dynamic modifications on lysine ubiquitination (+114.1 Da) and methionine oxidation (+16 Da). MS2 spectra were filtered (Xcorr>2) for doubly charged peptides and Xcor>3 for triply charged peptides and peptide probability<0.01) to tentatively identify TAK1 K72 ubiquitination and ubiquitin Lys48 linkages. These spectra were then manually inspected.

2.6 Quantitative real-time PCR

Total cellular RNA was isolated by TRIzol Reagent (Invitrogen) and 1 μ g of RNA was used for each RT-PCR reaction. Quantitative real-time PCR was performed using specific primers and QTaq One-Step qRT-PCR SYBR kit (Clontech). Reactions were analyzed using an Applied Biosystems 7300 real time PCR system. Data were normalized to housekeeping GAPDH gene and the relative abundance of transcripts was calculated by the Ct models.

2.7 Enzyme-linked immunosorbent assay (ELISA)

pBabe-vector, TAK1 wild-type and K72R mutant reconstituted TAK1-deficient MEF cells were either untreated or treated with mouse TNF α (2 ng/ml) for the indicated time points. Protein supernatants were collected after 15,000 g centrifuge for 15 min at 4°C. Mouse IL-6 concentrations in the supernatants were determined by ELISA (BD Biosciences) according to the manufacturer's instruction.

2.8 Luciferase reporter assay

Cells were seeded at 3×10^5 cells per well and cultured overnight in 6-well plates. The cells were transfected with NF- κ B-dependent *firefly* luciferase construct, *Renilla* luciferase and expression vectors. Luciferase activity was measured according to the manufacturer's protocol (Promega). The relative luciferase activity was calculated by dividing the *firefly* luciferase activity and the *Renilla* luciferase activity. All the data represent three independently repeated biological experiments performed in triplicate.

3. Results

3.1 TNF α induces TAK1 Lys⁴⁸-linked polyubiquitination and degradation at the later time course

We previously found that in response to TNF α , TAK1 is quickly activated by Lys⁶³-linked polyubiquitination[18]. Interestingly, we also observed that TAK1 is modified by Lys⁴⁸-linked polyubiquitination at the later stage (more than 30 mins after TNF α stimulation) of its activation [18]. This observation prompts us to further investigate Lys⁴⁸-linked polyubiquitination of TAK1. We stably re-introduced Flag-TAK1 wild-type into TAK1-deficient MEF cells and treated these cells with TNF α at different time points over a course of 2 hours in the presence of MG132. As expected, we found that TNF α induced TAK1 polyubiquitination in the presence of MG132, while without TNF α stimulation, MG132 alone was not sufficient to induce TAK1 polyubiquitination (Fig. 1a). TAK1

polyubiquitination induced by TNF α and MG132 is Lys48-linked because it is specifically recognized by Lys⁴⁸- but not Lys⁶³- specific antibodies (Fig. 1a). The time-dependent accumulation of TAK1 Lys⁴⁸-linked polyubiquitination indicates that it mediates TAK1 degradation. Next we examined whether TNF α could induce TAK1 degradation in HeLa cells. We incubated the cells with cycloheximide (CHX) for 2 hours to block protein synthesis. Consistent with the observation that TNF α could induce TAK1 polyubiquitination, TNF α promoted degradation of a portion of TAK1 protein in one hour of treatment (Fig. 1b). In addition, we also found that TNF α could slightly induce IKK β degradation and potentially trigger the degradation of TNFR1 as well as I κ B α upon 1-hour treatment of TNF α . (Fig. 1b-c). Together, our results indicate that TNF α induces TAK1 polyubiquitination and degradation at the later time course of stimulation.

3.2 Identification of TAK1 lysine-72 as a Lys⁴⁸-linked polyubiquitination receptor site by mass spectrometry

To obtain the direct evidence that TAK1 is ubiquitinated by Lys⁴⁸-linked polyubiquitination and to map the precise lysine ubiquitination site(s), we immunoprecipitated the Flag-TAK1 under partial protein denaturing conditions (see materials and methods for details), separated the immunoprecipitates by SDS PAGE, digested in gel by trypsin and employed mass spectrometry for detection of lysine ubiquitination site(s). To accumulate primarily the Lys48-linked polyubiquitinated TAK1 conjugates, HEK-293T cells were treated by MG132 for 2 hours before harvesting (Fig. 2a). At higher molecular weight region where polyubiquitinated TAK1 was supposed to be accumulated, we were able to identify multiple peptides of Lys⁴⁸ poly-Ub linkages (Fig. 2b) as well as an ubiquitinated TAK1 peptide that is conjugated at lysine 72 by diGlycine isopeptide bond (ubiquitination signature) using mass spectrometry (Fig. 2c). We were unable to detect any other polyUb linkage in the TAK1 immunoprecipitates under such condition, indicating that K48 polyUb is likely the dominant form of polyUb chain that targets primarily at K72 position. Based on the crystal structure [35], TAK1 lysine-72 localizes on the surface of the protein where the ubiquitination conjugation likely occur (Fig. S1). These results suggest that TAK1 is modified by Lys⁴⁸-linked polyubiquitination at lysine-72 which may play a role in modulating TAK1 activity.

3.3 TAK1 K72R mutation abolishes Lys⁴⁸-linked TAK1 polyubiquitination and enhances persistent TAK1 activation

To confirm that TAK1 is modified by Lys⁴⁸-linked polyubiquitination, we co-transfected FLAG-TAK1 into HEK-293T cells with HA-ubiquitin wild-type, Lys⁴⁸ or Lys⁶³ only mutants. Transfected cells were treated with/without MG132 for proteasome inhibition. Flag-TAK1 was then immunoprecipitated from different transfected cell lines and blotted with anti-HA, anti-Lys⁴⁸ and anti-Lys⁶³ specific ubiquitin antibodies for the detection of ubiquitination smears. Consistent with MS data, we found that TAK1 was modified by Lys⁴⁸-linked polyubiquitination and the ubiquitination TAK1 was accumulated upon proteasome inhibition (Fig. 3a).

To ascertain that lysine-72 is the major ubiquitination conjugation site for TAK1 Lys⁴⁸-linked polyubiquitination, we made a TAK1 mutant in which the lysine-72 is mutated to arginine (K72R) by site directed mutagenesis. Flag-TAK1 wild-type or K72R mutant along with HA-ubiquitin were transiently transfected into HEK-293T cells. Ubiquitinated TAK1 was then immunoprecipitated by anti-Flag antibodies and detected by anti-HA antibodies. As shown in Fig. 3b, this point mutation virtually completely abolished MG132-dependent TAK1 polyubiquitination. Together with the MS result, our data suggest that lysine-72 is the major lysine ubiquitination site for Lys⁴⁸-linked TAK1 polyubiquitination.

To test if lysine-72 is indeed a functional ubiquitination site, we transiently transfect TAK1 K72R mutant and TAB1 as well as NF- κ B luciferase reporter constructs into 293T cells. As shown in Fig. 3c, overexpression of K72R mutant induced a higher NF- κ B-dependent luciferase reporter activity compared to that of TAK1 wild-type. Together, these results indicate that Lys⁴⁸-linked TAK polyubiquitination at lysine-72 negatively regulate TAK1 function.

3.4 TAK1 lysine-72 mediates TNF α -induced Lys⁴⁸-linked TAK1 polyubiquitination and degradation

To test the hypothesis that lysine-72 polyubiquitination of TAK1 negatively regulates TAK1 activation in the later time point of TNF α stimulation, we stably re-introduced Flag-TAK1 wild-type and K72R mutant into TAK1-deficient MEF cells and treated these two cells with TNF α at four consecutive time points (at 30, 60, 90 and 120 mins) in the presence of MG132. As shown in Fig. 4a, polyubiquitination of wild type TAK1 were potently induced after 30 minutes of TNF α stimulation. However, similar scenario did not replicate in the K72R mutant cells, in which no detectable TAK1 polyubiquitination induction and accumulation were observed.

To test whether this polyubiquitination at lysine-72 represents the signal for TAK1 protein degradation, we treated either TAK1 wild-type- or K72R mutant-reconstituted MEF cells with TNF α at different time points in the presence of CHX to inhibit TAK1 protein biosynthesis. Consistently, we found that TNF α -induced TAK1 degradation was inhibited in TAK1 K72R mutant cells comparing to that of wild type cells. The protein level of wild type TAK1 but not K72R was appreciably reduced in 6 hours of TNF α stimulation (Fig. 4b-c). These results suggest that TAK1 Lysine-72 residue mediates TNF α -induced TAK1 polyubiquitination and degradation at the later stage of TNF α stimulation.

3.5 TAK1 K72R mutant prolongs TNF α -induced NF- κ B activation

To further characterize the functional role of TAK lysine-72-mediated polyubiquitination in the context of TNF α -mediated NF- κ B pathway, we probed the TNF α -induced IKKs phosphorylation, JNK phosphorylation, I κ B α phosphorylation, I κ B α degradation, and RelA nuclear translocation as markers for the activation of the pathway. TNF α -induced phosphorylation levels of IKKs, JNK and I κ B as well as RelA nuclear presence are considerably higher and last longer in TAK1 K72R mutant-reconstituted cells compared to the wild-type counterpart (Fig. 5a-b). Consistent with Figure 3c, we also found that TAK1 K72R point mutation enhanced TNF α -induced NF- κ B luciferase activity compared to that of TAK1 wild-type (Fig. 5c). Taken together, these results indicate that TAK1 polyubiquitination at lysine-72 is functional and might be important for preventing excessive TNF α -induced NF- κ B activation, which could be detrimental to the cells.

3.6 TAK1 polyubiquitination at lysine-72 attenuates TNF α -induced IL-6 gene expression

TNF α induces IL-6 gene expression via NF- κ B activation. To further examine the role of TAK1 polyubiquitination at lysine-72 in TNF α -induced NF- κ B targeted gene expression, total RNAs were isolated from either TAK1 wild-type or K72R mutant stable MEF cell lines with or without TNF α stimulation and analyzed by quantitative RT-PCR. In this assay, we found that TNF α induced a higher level of IL-6 gene expression and IL-6 protein production in the TAK1 K72R mutant-reconstituted cells compared to the TAK1 wild-type-reconstituted cells (Fig. 6a-b). These data suggest that polyubiquitination of TAK1 at lysine-72 is required for preventing excessive TNF α -induced NF- κ B activation and cellular responses.

4. Discussion

Deregulation of immune responses can be extremely harmful to the host cells. As a master transcription factor in immunity, NF- κ B activity must be tightly controlled. TAK1, a member of the MAPKKK family, was originally found to function in the transforming growth factor- β (TGF- β)-mediated MAPK activation [36]. TAK1 has been further demonstrated to be an essential component in TNF α -mediated activation of NF- κ B and JNK [37-39]; Post-translational regulation of TAK1, especially via phosphorylation, has been extensively investigated. For example, it has been suggested that after its own activation, TAK1 is dephosphorylated and subsequently inactivated by phosphatases such as PP2A and PP6 [40, 41]. However, whether TAK1 is modulated by the ubiquitin/proteasome pathway is unclear. In this report, we found that TAK1 is modified by Lys⁴⁸-linked polyubiquitination and degraded by ubiquitin proteasome system to downregulate TAK1-mediated NF- κ B activation. Furthermore, we provided evidence that TAK1 lysine 72 is the major site for TAK1 Lys⁴⁸-linked polyubiquitination. Lastly, we presented data to show that this modification is functional and important for the inhibition of the pathway. Thus, our data demonstrate that ubiquitin/proteasome pathway-mediated TAK1 degradation plays a critical role in the downregulation of TNF α -induced NF- κ B activation.

Innate immunity provides an evolutionarily conserved barrier for higher eukaryotes to battle against infectious microorganisms. Genetic approaches in *Drosophila* have uncovered two distinct pathways that are mediated by Toll and Imd. TAK1 acts as an activator of both the JNK and the Relish in Imd signaling. It has been suggested that proteasomal degradation of TAK1 by an E3 ligase POSH could lead to rapid termination of JNK activation in *Drosophila* cells [42]. Two other ubiquitin E3 ligases have been proposed to target TAK1 for ubiquitin-proteasome degradation in mammalian system, although the detailed molecular mechanism for such regulation is still murky [43]. RNF142/SH3RF1 is a human homolog of fly POSH protein. Whether or not RNF142 is the primary E3 ligase for TAK1 K72 ubiquitination shall be tested in the future.

Ubiquitination via different linkages may play distinct roles [29, 44]. While Lys⁴⁸-linked polyubiquitination is known for substrate degradation, Lys⁶³-linked polyubiquitination regulates non-proteolysis functions of the substrates such as control of kinase activity in the signaling transduction pathway [9]. For instance, recruitment of endogenous RIP1 to TNFR1 is initiated by the transient Lys⁶³-linked polyubiquitination of RIP1 which is then followed by Lys⁴⁸-linked polyubiquitination to terminate the signal transduction pathway [30]. While we could not see obvious TNF α -induced RIP1 degradation after 90 minutes of TNF α treatment, we did observe a minor yet truly detectable amount of TNF α -induced degradation of TAK1 and IKK β . It is likely that the small portion of degradable TAK1 proteins are the activated TAK1 during signal transduction and degradation of the activated TAK1 is critical for the termination of TAK1-mediated signaling. Indeed, similar phenomenon of “ubiquitin editing” was observed by in IL-1 β -induced IRAK1 polyubiquitination [45]. In TNF α -mediated signaling, Lys⁶³-linked polyubiquitination of TAK1 is required for its activation while Lys⁴⁸-linked polyubiquitination of TAK1 leads to its degradation. It is highly likely that such ubiquitin editing mechanism is common for key molecules in TNF α -induced NF- κ B activation.

In summary, we present evidence to suggest that Lys⁴⁸-linked TAK1 polyubiquitination at lysine-72 negatively regulates TNF α -induced NF- κ B activation. In view of the data presented here and in previous reports, we propose a working model (Fig. 7) in which, upon TNF α binding to its receptor, TNF α receptor-mediated signaling events lead to Lys⁶³-linked TAK1 polyubiquitination at lysine-158 and its immediate activation. After activation,

TAK1 is further modified by Lys⁴⁸-linked polyubiquitination at lysine-72 to downregulate TAK1-mediated NF- κ B activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

TNF α induces TAK1 Lys⁴⁸-linked polyubiquitination and degradation at the later time course of stimulation. (a) TNF α induces TAK1 Lys⁴⁸-linked polyubiquitination. TAK1-deficient MEF cells with the stable expression of FLAG-TAK1 wild-type were either untreated or treated with TNF α (10 ng/ml) for the time points indicated in the presence of MG132 and subsequently lysated. FLAG-TAK1 proteins in the cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-ubiquitin, anti-FLAG, anti-Lys⁴⁸ specific ubiquitin and anti-Lys⁶³ specific ubiquitin antibodies to detect the presence of ubiquitinated FLAG-TAK1. (b) TNF α induced TAK1 degradation. HeLa cells were first treated with 2 hours of CHX and further treated with TNF α for the time points indicated. Cell lysates were immunoblotted with indicated antibodies. (c) Quantification of B. * $P < 0.05$, ** $P < 0.01$, versus controls, t -test. Results from (a) and (b) are representative of three independent experiments. All experiments were performed at least three times with similar results.

Fig. 2.

TAK1 Lys⁴⁸-linked polyubiquitination at lysine-72 detected by mass spectrometry. (a) A SDS-PAGE gel image of Flag-TAK1 immunoprecipitation. Five 10 cm dishes of 293T cells were transiently transfected with FLAG-TAK1 expression vector. Transfected cells were treated with MG132 (10 ng/ml) for 2 hours and subsequently lysed. FLAG-TAK1 proteins in the cell lysates were immunoprecipitated with anti-FLAG antibodies and separated by SDS-PAGE. (b) The MS/MS spectrum of K48 polyUb linkage. (c) The MS/MS spectrum of a peptide derived from polyubiquitinated TAK1.

Fig. 3.

TAK1 K72R mutant abolishes Lys⁴⁸-linked TAK1 polyubiquitination and enhances NF- κ B activation. (a) TAK1 overexpression induces TAK1 Lys⁴⁸-linked polyubiquitination. Expression vectors encoding FLAG-TAK1 was co-transfected into HEK-293T cells with control vector and expression vectors encoding HA-ubiquitin wild-type, Lys⁶³-only and Lys⁴⁸-only, respectively. Transfected cells will treated with or without MG132 (10 ng/ml) for 2 hours and subsequently lysed. FLAG-TAK1 proteins in the transfected cells were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-HA antibodies to detect the presence of the ubiquitinated FLAG-TAK1. (b) Overexpression of TAK1 induces TAK1 polyubiquitination at Lys-72. Expression vectors encoding HA-ubiquitin was

co-transfected into HEK-293T cells with control vector and expression vectors encoding FLAG-TAK1 wild-type and lysine-72 to arginine (K72R) mutant, respectively. FLAG-TAK1 proteins in the transfected cells were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-HA antibodies to detect the presence of ubiquitinated FLAG-TAK1. (c) The effect of TAK1 K72R mutation on TAK1/TAB1-induced NF- κ B activation. TAB1 expression vectors, NF- κ B luciferase reporter and control *Renilla* luciferase reporter vectors were co-transfected into 293T cells with empty vector or expression vectors encoding TAK1 wild-type and K72R mutant, respectively. The relative luciferase activity was measured 48 h later and normalized with the *Renilla* activity. Error bars indicate $\pm$ standard deviation in triplicate experiments. Data from (c) are plotted as means $\pm$ SD; * $P < 0.05$, versus controls, t-test. Results from (a) and (b) are representative of three independent experiments. All experiments were performed at least three times with similar results.

Fig. 4.

TAK1 K72R mutation inhibits TNF α -induced TAK1 Lys⁴⁸-linked polyubiquitination and degradation. (a) TNF α induces TAK1 Lys⁴⁸-linked polyubiquitination at Lys-72. TAK1-deficient MEF cells with stable expression of FLAG-TAK1 wild-type and K72R mutant were either untreated or treated with TNF α (10 ng/ml) for the time points indicated in the presence of MG132 and subsequently lysed. FLAG-TAK1 proteins in the cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-ubiquitin antibodies to detect the presence of ubiquitinated FLAG-TAK1. (b) TAK1 Lys-72 is required for TNF α -induced TAK1 degradation. TAK1-deficient MEF cells with stable

expression of TAK1 wild-type and K72R mutant were either untreated or treated with TNF α (10 ng/ml) for the time points indicated in the presence of CHX and subsequently lysed. The whole cell lysates were immunoblotted with anti-TAK1 antibodies to detect the stability of TAK1. (c) Quantification of results from (b). * $P < 0.05$, versus controls, t -test. Results from (a) and (b) are representative of three independent experiments. All experiments were performed at least three times with similar results.

Fig. 5.

TAK1 K72R mutation prolongs TNF α -induced NF- κ B and JNK activation. (a) TAK1 K72R mutant prolongs TNF α -induced IKK activation, JNK activation and I κ B α degradation. TAK1-deficient MEF cells with stable expression of TAK1 wild-type and K72R mutant were either untreated or treated with TNF α (10 ng/ml) for the time points indicated and subsequently lysed. The whole cell lysates were immunoblotted with antibodies indicated to detect IKK phosphorylation, JNK phosphorylation, I κ B α phosphorylation and I κ B α degradation. (b) TAK1 K72R mutant prolongs TNF α -induced RelA translocation. TAK1-deficient MEF cells with stable expression of TAK1 wild-type and K72R mutant were either untreated or treated with TNF α (10 ng/ml) for the time points indicated and subsequently

harvested. The nuclear extracts were prepared and subjected to SDS-PAGE. Nuclear RelA was determined by immunoblotting with an anti-RelA antibody, and PCNA was detected as a loading control. (c) TAK1 Lysine-72 is required for the downregulation of TNF α -induced NF- κ B activation. NF- κ B luciferase reporter and *Renilla* luciferase plasmids were co-transfected into TAK1 wild-type and K72R mutant reconstituted MEF cells, and then the cells were either untreated or treated with TNF α (2 ng/ml) for 6 h. The relative luciferase activity was measured and normalized with the *Renilla* activity. Error bars indicate $\pm$ standard deviation in triplicate experiments. Data from (c) are plotted as means $\pm$ SD; * P<0.05, versus controls, *t*-test. Results from (a) and (b) are representative of three independent experiments. All experiments were performed at least three times with similar results.

Fig. 6.

TAK1 K72R mutation enhances TNF α -induced IL-6 gene expression. (a) TAK1 K72R mutation enhances TNF α -induced NF- κ B dependent IL-6 gene expression. TAK1 wild-type and K72R mutant reconstituted MEF cells were either untreated or treated with TNF α (2 ng/ml) for the time points indicated. Total RNAs from these cells were harvested. IL-6 transcript levels in the cells were measured using quantitative RT-PCR normalized to GAPDH. The data is presented as the average of three separate experiments with standard deviation. (b) TAK1 K72R mutation enhances the TNF α -induced IL-6 production. TAK1 wild-type and K72R mutant reconstituted MEF cells were either untreated or treated with TNF α (2 ng/ml) for the time points indicated. The supernatants from these cell cultures were collected and subjected to the mouse IL-6 ELISA analysis according to the manufacturer's instruction. Data from (a) and (b) are plotted as means $\pm$ SD; * $P < 0.05$, ** $P < 0.01$, versus controls, t -test.

Fig. 7.

A working model for the role of TAK1 lysine-72 in the negative regulation of TNF α -induced NF- κ B activation. Upon TNF α binding to its receptor, TNF α receptor-mediated signaling events lead to Lys⁶³-linked TAK1 polyubiquitination at lysine-158 and its activation. After activation, TAK1 is further modified by Lys⁴⁸-linked polyubiquitination at lysine-72. Lys⁴⁸-linked polyubiquitinated TAK1 is then degraded via ubiquitin/proteasome pathway to downregulate TAK1 mediated NF- κ B activation.