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The Zinc Finger Mutation C417R of I-κB Kinase γ Impairs Lipopolysaccharide- and TNF-Mediated NF-κB Activation through Inhibiting Phosphorylation of the I-κB Kinase β Activation Loop[1]

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The Zinc Finger Mutation C417R of I-κB Kinase γ Impairs Lipopolysaccharide- and TNF-Mediated NF-κB Activation through Inhibiting Phosphorylation of the I-κB Kinase β Activation Loop[1]

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The activation of the I-κB kinase (IKK) complex by TNF or LPS stimulates phosphorylation and degradation of I-κBα, leading to the nuclear translocation of NF-κB. The IKK complex is mainly composed of two catalytic subunits, IKKα and IKKβ, and a chaperon subunit IKKγ. Although IKKγ does not have catalytic activity, it is essential for IKK activation induced by multiple stimuli. Importantly, the key residue cysteine 417 at the zinc finger domain of IKKγ has been found to be mutated to arginine (IKKγC417R) in a human genetic disorder called the anhydrotic ectodermal dysplasia with immunodeficiency. To understand the underlying mechanisms of immunodeficiency, we examined whether the IKKγC417R mutant modified IKK activation and NF-κB transcription stimulated by LPS or TNF in human monocytes. We found that overexpression of IKKγC417R severely impaired LPS- and TNF-induced I-κBα phosphorylation and degradation in a dominant-negative fashion. Also, LPS- and TNF-induced NF-κB transcription was inhibited by IKKγC417R. The reconstitution of IKKγ, but not IKKγC417R, in IKKγ-deficient cells restored NF-κB signaling, indicating the zinc finger structure of IKKγ plays a key role in IKK activation. Moreover, C417R mutation in IKKγ abolished both LPS- and TNF-induced phosphorylation of the activation loop of IKKβ. Collectively, our results indicated that the zinc finger structure of IKKγ plays a key role in LPS- and TNF-induced NF-κB activation. The anhydrotic ectodermal dysplasia with immunodeficiency patients' immunodeficiency may be associated with NF-κB defect in response to bacterial stimulation. ***The Journal of Immunology,*** **2004, 172: 2446–2452.**

Nuclear factor-κB plays a critical role in the regulation of inflammation, immunity, cell survival, and proliferation (1–4). NF-κB was originally identified as a transcription activator that bounds to a specific DNA motif (GGGGACTTCCC) in the intronic enhancer of the Ig κ L chain gene in B cells. Subsequently, it was found that NF-κB was a ubiquitous cellular factor that was retained in the cytoplasm by inhibitory proteins I-κBs. NF-κB consists of homo- and heterodimeric complexes of members of the Rel family of proteins, including p50, p52, p65/RelA, c-Rel, and RelB. In mammalian cells, the most widely distributed κB-binding activity is a heterodimer of p50 and p65/RelA proteins, in which the p65/RelA subunit has potent *trans* activation activity. The NF-κB-inhibitory proteins, I-κBs, consist of I-κBα, I-κBβ, and I-κBε (1–4). Upon stimulation by LPS or TNF, the I-κB kinase (IKK)[4] complex is activated, resulting in the phosphorylation of I-κBs on two conserved N-terminal serine residues. The phosphorylated I-κBs are ubiquitinated and subsequently degraded by the 26S proteasome, thereby liberating NF-κB to enter the nucleus to activate gene expression (1, 3, 4).

Biochemical and genetic studies have demonstrated that the IKK complex is critical for NF-κB activation induced by LPS and proinflammatory cytokines. The IKK complex is mainly composed of two catalytic subunits, IKKα and IKKβ, and IKKγ (also known as NF-κB essential modulator), a scaffold molecule without known catalytic activity (4). Gene depletion studies demonstrate that IKKβ, but not IKKα, plays an essential role in NF-κB activation mediated by LPS and proinflammatory cytokines (5–9). Although IKKγ appears to lack catalytic function, it plays a critical role in assembling the IKK complex. Gene knockout experiments have demonstrated that IKKγ is essential for IKK activation induced by proinflammatory cytokines and LPS (4, 10–13). Importantly, mutations in the human *IKKγ* locus have been found to be responsible for a human X-linked genetic disorder known as incontinentia pigmenti (IP), an X-linked dominant and male-lethal disorder (14).

IKKγ contains several predicted functional domains, including two coiled-coil regions that are separated by α helices, a leucine zipper motif and a putative zinc finger domain at the C terminus. The C terminus of IKKγ has been found to play a regulatory role in IKK activation (4, 10–14). Recently, Doffinger et al. (15) reported that mutations at the C terminus of IKKγ were associated with human anhydrotic ectodermal dysplasia with immunodeficiency (EDA-ID). They demonstrated that stop codon mutations at IKKγ caused EDA-ID with osteopetrosis and lymphoedema by

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[4] Abbreviations used in this paper: IKK, I-κB kinase; CD40L, CD40 ligand; EDA-ID, anhydrotic ectodermal dysplasia with immunodeficiency; HA, hemagglutinin; IP, incontinentia pigmenti; TRAF, TNFR-associated factor; XHM-ED, hyperimmunoglobulin M syndrome and hypohydrotic ectodermal dysplasia.

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inhibiting NF-κB signaling. In addition, some EDA-ID patients also had the C417R missense mutation at the zinc finger domain of IKKγ. Jain et al. (16) identified that the C417R mutation is associated with a human genetic disease called X-linked primary immunodeficiency, characterized by hyperimmunoglobulin M syndrome and hypohydrotic ectodermal dysplasia (XHM-ED). C417 is a predicted key residue for maintaining the C-terminal zinc finger motif structure of IKKγ. Because NF-κB plays a critical role in immune responses, Jain et al. (16) examined whether the IKKγC417R mutant modified NF-κB activation mediated by LPS, TNF, and CD40 ligand (CD40L). They found that CD40L could not activate NF-κB in the cells derived from the affected patients, whereas LPS- and TNF-mediated NF-κB signaling was intact. However, paradoxically, TNF secretion by these cells was significantly reduced following treatments with LPS and IFN γ, both of which are well-known activators of NF-κB.

Given the fact that TNF is transcriptionally regulated by NF-κB (1–4) and that the XHM-ED and EDA-ID patients often die of severe bacterial infection (14–16), it is unlikely that the CD40 signaling defect is solely responsible for the patients' immunodeficiency. Furthermore, although several studies have suggested that the IKKγC417R mutant inhibits NF-κB activation, these studies were performed in fibroblasts and embryonic mouse fibroblasts (4). Also, it is unclear how the zinc finger mutation affects IKK activation and NF-κB transcription induced by LPS and TNF. In this study, we extensively examined whether the IKKγC417R mutant had effects on LPS- or TNF-mediated NF-κB activation. We found that IKKγC417R impaired both TNF- and LPS-induced IKK activation and NF-κB transcription in a dominant-negative fashion. Consistently, we found that NF-κB-dependent IL-8 expression was inhibited by overexpression of IKKγC417R. Furthermore, human THP.1 monocytes expressing IKKγC417R were more sensitive to LPS- and TNF-mediated apoptosis than wild-type cells due to NF-κB dysfunction. Our results demonstrate that the zinc finger structure of IKKγ is critical for LPS- and TNF-induced NF-κB signaling and provide new insights into the mechanism of IKK activation.

Materials and Methods

Cell culture and retroviral infection

Human THP.1 monocytes and Jurkat T cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Invitrogen, San Diego, CA) supplemented with 10% FBS, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. LPS from *Escherichia coli* 055:B5 was purchased from Sigma-Aldrich (St. Louis, MO), and LPS from *Porphyromonas gingivalis* 7346 was provided by R. Arnold at University of North Carolina (Chapel Hill, NC). To establish cell lines stably expressing wild-type IKKγ or IKKγC417R, a retrovirus expression system was used, as described previously (17). Briefly, retroviruses were generated by transfecting the retroviral vector encoding IKKγ or IKKγC417R (18) into 293T cells by the calcium phosphate method. Retrovirus-containing supernatant was harvested 48 h later, filtered, and stored in −70°C. Cells were infected with retroviruses in the presence of 6 μg/ml polybrene (Sigma-Aldrich). Forty-eight hours after infection, cells were treated with G418 (600 μg/ml) for 2 wk. The resistant cells were pooled, and cells expressing IKKγ or IKKγC417R were confirmed by Western blot analysis.

Western blot analysis

Human THP.1 monocytes or Jurkat T cells were treated with *E. coli* LPS (100 ng/ml), *P. gingivalis* LPS (500 ng/ml), or TNF (20 ng/ml), then harvested and washed once with PBS. Cells were pelleted and lysed with cell lysis buffer containing 1% Nonidet P-40, 5% sodium deoxycholate, 1 mM PMSF, 100 mM sodium orthovanadate, and 1/100 protease inhibitor cocktails (Sigma-Aldirich). The protein concentration was determined according to the manufacturer's protocol (Bio-Rad). Whole cell lysates were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) using a semidry gel transfer cell. The membranes were blocked with 5% nonfat milk overnight at 4°C and probed with the primary Abs. The immunocomplexes were visualized with HRP-coupled goat anti-rabbit or anti-mouse IgG (Promega, Madison, WI) using the SuperSignal reagents (Pierce, Rockford, IL), as described previously (17–21). The primary Abs were from the following sources: anti-IKKα, anti-IKKβ, anti-p65, and anti-I-κBα polyclonal Abs from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-p65 (serine 536), anti-phospho-I-κBα (serine 32), and anti-phospho-IKKβ (Ser^{181}) Abs from Cell Signaling (Beverly, MA).

Northern blot analysis

Human THP.1 monocytes were treated with LPS and then lysed with TRIzol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's protocol. Aliquots (15 μg) of RNA samples were separated on a 1.4% agarose-formaldehyde gel and transferred onto a nylon filter for 16–24 h (Bio-Rad). RNA was cross-linked with a UV cross-linker (Promega). Blots were prehybridized with PerfectHyb (Sigma-Aldrich) buffer containing salmon sperm DNA (100 μg/ml) for 20 min and then hybridized with PerfectHyb buffer containing radiolabeled IL-8 cDNA probes. The probes were generated with a random-primed labeling kit (Amersham, Arlington Heights, IL) in the presence of [α-^{32}P]dCTP (ICN Pharmaceuticals, Costa Mesa, CA). The probes were purified with a microG50 Sephadex column (Amersham). After hybridization, the blots were washed twice in 2× SSC-0.1% SDS for 10 min at room temperature and twice in 0.1× SSC-0.1% SDS for 20 min at 42°C, as described previously (22).

Plasmids and NF-κB luciferase reporter assay

The mutant IKKγC417R was prepared using PCR-based mutagenesis (18). Cells were cotransfected with 2× NF-κB luciferase reporter plasmids with IKKγ or IKKγC417R expression vectors using lipofectamine reagents, according to the manufacturer's instructions (Invitrogen). Cells were cotransfected with the pRL-TK *Renilla* luciferase reporter for normalizing transfection efficiency. The luciferase activities were measured with a dual luciferase system (Promega).

Electrophoretic mobility shift assays (EMSA)

Cells were treated with TNF or LPS for the different time periods, then washed with ice-cold PBS and collected. Nuclear extracts were prepared, as described previously (20–23). Aliquots (5 μg) of cell nuclear extracts were preincubated with 1 μg of poly(dI-dc) in binding buffer (10 mM Tris (pH 7.7), 50 mM NaCl, 20% glycerol, 1 mM DTT, 0.5 mM EDTA) for 10 min at room temperature. Approximately 1.5×10^4 cpm of ^{32}P-labeled DNA probe containing the class I MHC NF-κB site (underlined) (5′-CAG GGC TGG GGA TTC CCC ATC TCC ACA GTT TCA CTT-3′) was then added, and reaction proceeded for 15 min. The complexes were resolved on a 5% polyacrylamide gel in Tris-glycine buffer consisting of 25 mM Tris, 190 mM glycine, and 1 mM EDTA at room temperature. The gel was dried at 80°C for 60 min and exposed to an x-ray film. To confirm DNA-binding specificity, nuclear proteins were preincubated with polyclonal Abs against NF-κB subunit p65/RelA for 10 min and then incubated with NF-κB DNA probe.

Results

Overexpression of IKKγC417R inhibits TNF-induced IKK activation in a dominant-negative fashion

Because the mutant IKKγC417R was found in XHM-ED or EDA-ID patients, first we wanted to know whether IKKγC417R could act as a dominant-negative mutant to inhibit TNF-mediated IKK activation and NF-κB transcription in human THP.1 monocytes. To prevent clonal variation, we used retrovirus-mediated transduction for establishing stable cell lines, as described previously (17, 18). Retroviruses encoding wild-type IKKγ or IKKγC417R were produced in 293T cells. Human THP.1 monocytes were infected with retroviruses expressing IKKγC417R or IKKγ and selected with G418 for 2 wk. As shown in Fig. 1*A*, human THP.1 monocytes expressing IKKγ (THP/IKKγ), IKKγC417R (THP/IKKγC417R), or empty vector (THP/V) were obtained, as determined by Western blot analysis. Overexpression of IKKγ or IKKγC417R had no effects on the level of IKKα, IKKβ, and p65. As shown in Fig. 1*B*, both IKK activity (I-κBα phosphorylation) and I-κBα degradation were inhibited in THP/IKKγC417R (*lanes 9–12*), but not in control cells THP/V (*lanes 1–4*) in response to TNF stimulation. Because the IKK complex

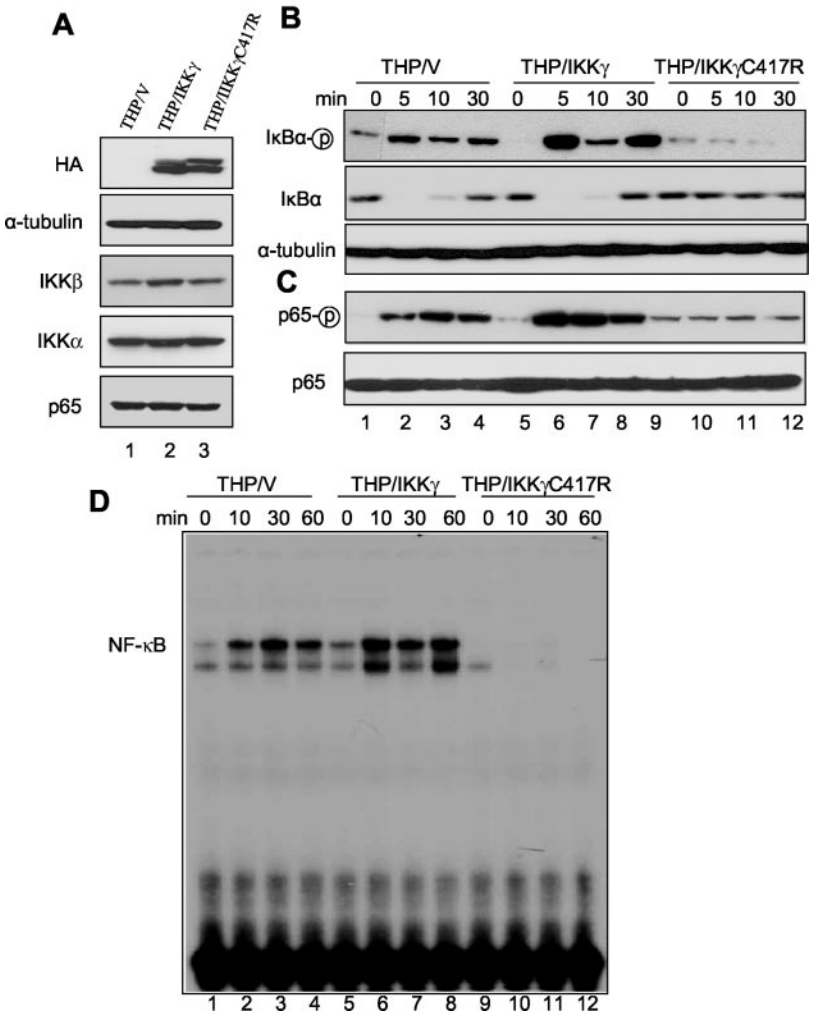

FIGURE 1. Overexpression of IKKγC417R inhibits TNF-induced NF-κB activation in human THP.1 monocytes. *A*, Human THP.1 monocytes expressing IKKγ or IKKγC417R. Cells were tranduced with retroviruses expressing HA-IKKγ, HA-IKKγC417R, or control vector and selected with G418 (600 μg/ml) for 2 wk. The whole cell proteins were extracted, as described in *Materials and Methods*. Aliquots (50 μg) of extracts were probed with anti-hemagglutinin (HA) mAbs (1/1000), anti-IKKβ (1/1000), anti-IKKα (1/1000), and anti-p65 (1/5000) polyclonal Abs. For loading control, the membrane was stripped and reprobed with anti-α-tubulin mAbs (1:5000). *B*, IKKγC417R inhibited TNF-induced phosphorylation and degradation of I-κBα. Cells were treated with TNF (20 ng/ml) for the indicated time periods. Aliquots (50 μg) of extracts were probed with anti-phospho-I-κBα Abs (1/1000) and anti-I-κBα Abs (1/500). For loading control, the membranes were stripped and reprobed with anti-α-tubulin. *C*, IKKγC417R inhibited IKK-mediated p65 phosphorylation induced by TNF. Aliquots (50 μg) of extracts were probed with anti-phospho-p65 Abs. For loading control, the membranes were stripped and reprobed with anti-p65 polyclonal Abs. *D*, IKKγC417R inhibited TNF-induced nuclear translocation of NF-κB. Cells were treated with TNF for the indicated time periods and the nuclear proteins were isolated, as described in *Materials and Methods*. Aliquots (5 μg) of nuclear proteins were incubated with ^{32}P-labeled cDNA probes for 10 min. The DNA-protein-binding complexes were resolved on a 5% polyacrylamide gel.

also phosphorylates the NF-κB subunit RelA/p65 at serine 536 (4, 19), we examined whether the IKK-mediated p65 phosphorylation induced by TNF was inhibited by IKKγC417R using anti-phospho-p65 (serine 536) Abs. As shown in Fig. 1*C*, there were significant increases in p65 phosphorylation in THP/V cells (*lanes 1–4*), but not in THP/C417R cells (*lanes 9–12*), following TNF stimulation. To determine whether IKKγC417R inhibited the nuclear translocation of NF-κB, nuclear proteins were isolated. As shown in Fig. 1*D*, TNF rapidly induced the nuclear translocation of NF-κB in THP/V cells, but not in THP/IKKγC417R cells (compare *lanes 1–4* with *lanes 9–12*). Because some studies found that overexpression of IKKγ could inhibit IKK activation (24), we examined whether NF-κB inactivation in THP/IKKγC417R cells was due to overexpression. As shown in Fig. 1, *B*, *C*, and *D*, conversely, overexpression of IKKγ enhanced TNF-induced I-κBα phosphorylation and degradation and the nuclear translocation of NF-κB in THP/IKKγ cells, confirming that the inhibition of NF-κB by IKKγC417R was not simply due to overexpression of IKKγ. Taken together, our results demonstrate that IKKγC417R can suppress IKK activity and NF-κB activation in a dominant-negative fashion in human THP.1 monocytes.

IKKγC417R inhibited LPS-induced IKK activation and NF-κB activation in a dominant-negative fashion

Patients with EDA-ID are highly susceptible to infections with multiple microorganisms. Toll receptor signaling, which generally signals through NF-κB, plays a critical role in inducing an immunological, antimicrobial response (25–28). Unlike TNF signaling, LPS binds to Toll-like receptor to activate NF-κB through TNFR-associated protein-6 (25). Therefore, we were also interested in knowing whether IKKγC417R impaired LPS-mediated NF-κB activation in a dominant-negative fashion in human THP.1 monocytes. Cells were treated with LPS from *E. coli* for the indicated time periods and then subjected to Western blot analysis. As shown in Fig. 2*A*, LPS-stimulated IKK activity (I-κBα phosphorylation) and I-κBα degradation were inhibited in THP/IKKγC417R cells (*lanes 9–12*), but not in THP/V cells (*lanes 1–4*). Similarly, IKK-mediated p65 phosphorylation induced by LPS was also abolished by IKKγC417R. Conversely, overexpression of IKKγ did not inhibit LPS-induced p65 phosphorylation and I-κBα phosphorylation and degradation. As shown in Fig. 2*B*, consistently, LPS-induced nuclear translocation of NF-κB was inhibited in THP/IKKγC417R cells (*lanes 5–8*), but not in THP/V cells (*lanes 1–4*). The specificity of NF-κB-binding activity was confirmed with polyclonal Abs against p65 by the supershift assay (Fig. 2*B*, *lane 9*). Also, we tested whether IKKγC417R impaired NF-κB activation induced by LPS from other pathogens. LPS from *P. gingivalis*, a common pathogen of chronic oral inflammatory diseases, has been found to induce chronic oral inflammation and inflammatory bone destruction (26). As shown in Fig. 3, overexpression of IKKγC417R also inhibited p65 phosphorylation and I-κBα phosphorylation and degradation induced by *P. gingivalis* LPS.

Next, we performed the NF-κB-dependent luciferase assay to determine whether IKKγC417R inhibited NF-κB transcription.

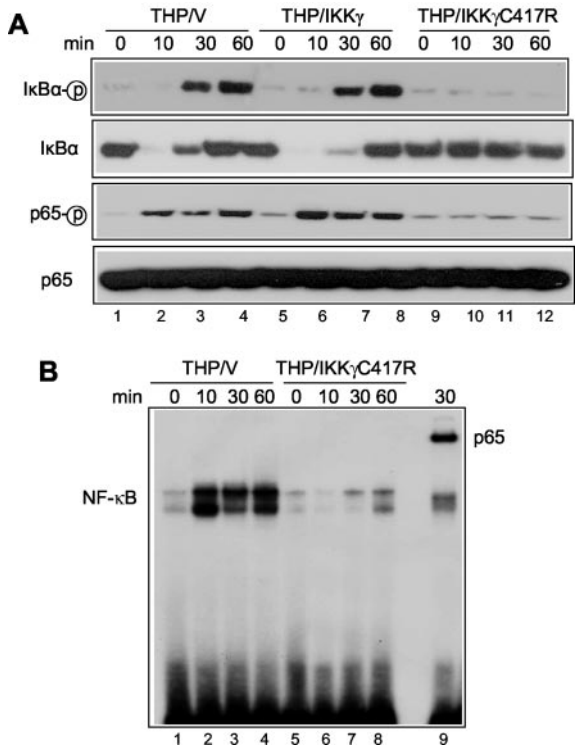

FIGURE 2. Overexpression of IKKγC417R inhibited *E. coli* LPS-induced NF-κB activation. *A*, IKKγC417R inhibited IKK activation induced by *E. coli* LPS. Cells were treated with *E. coli* LPS (100 ng/ml) for the indicated time points, and Western blot analysis was performed, as described in Fig. 1*B*. *B*, IKKγC417R suppressed LPS-induced nuclear translocation of NF-κB. Cells were treated with *E. coli* LPS for the indicated time periods, and nuclear proteins were isolated. The gel-shift assay was performed, as described in Fig. 1*C*. For the supershift assay, nuclear proteins for LPS-treated THP/V cells (*lane 2*) were preincubated with anti-p65 polyclonal Abs for 10 min.

Because TNF rapidly induces apoptosis under the inhibition of NF-κB, LPS-mediated NF-κB transcription was examined. Human THP.1 monocytes were cotransfected with 2× NF-κB-dependent luciferase reporter and IKKγC417R expression vector or control vector. Twenty-four hours after transfection, cells were treated with LPS for short periods (8 h) before LPS-mediated apoptosis was observed. As shown in Fig. 4*A*, LPS-induced NF-κB transcriptional activity was significantly inhibited by overexpression of IKKγC417R. To further verify our results, we also performed the Northern blot analysis to directly examine whether the expression of IL-8, which is an NF-κB-inducible gene, was suppressed in THP.1 cells stably expressing IKKγC417R cells after LPS treatment. As shown in Fig. 4*B*, the level of IL-8 expression was significantly reduced in THP/IKKγC417R cells compared with THP/V cells in response to LPS stimulation.

IKKγC417R increased cell sensitivity to both LPS and TNF by inhibiting NF-κB

We and others have previously found that NF-κB plays an essential role in the inhibition of TNF-mediated apoptosis (2, 20, 21). Inhibition of NF-κB renders cells sensitive to TNF killing. IKK$\gamma^{-/-}$ mouse fibroblasts and human fibroblasts from IP patients are sensitive to TNF-mediated apoptosis (4, 14). Thus, we examined whether IKKγC417R abolished NF-κB-mediated survival function in human monocytes. As shown in Fig. 5*A*, there were significantly more cell deaths in THP/IKKγC417R cells than in THP/V cells following TNF stimulation. Several studies reported

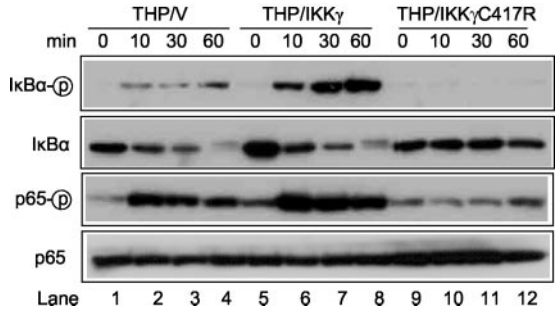

FIGURE 3. IKKγC417R inhibited *P. gingivalis* LPS-induced IKK activity. Cells were treated with *P. gingivalis* LPS (500 ng/ml) for the indicated time points, and Western blot analysis was performed, as described in Fig. 1*B*.

that LPS also induced monocyte apoptosis through the Fas-associated death domain-containing protein-dependent mechanism (27–29). Because the percentage of cell death induced by LPS in human THP.1 monocytes was relatively low, the cell death ELISA, which accurately measured DNA fragmentation and histone release from apoptotic cells, was used. As shown in Fig. 5*B*, there were more apoptotic cells in THP/IKKγC417R cells than in THP/V cells following LPS treatment. These results demonstrate

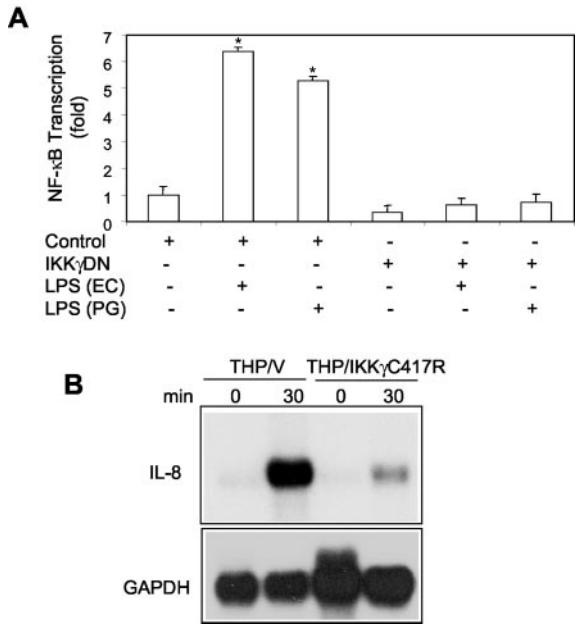

FIGURE 4. Overexpression of IKKγC417R inhibits LPS-induced NF-κB transcription. *A*, Human THP.1 monocytes were cotransfected with 2× κB luciferase reporter, the indicated IKKγC417R expression vectors, or control empty vector. Cells were cotransfected with pRK-*Relina* for the internal normalization. Twenty-four hours after transfection, cells were treated with LPS for 8 h. LPS (EC), *E. coli* LPS; LPS (PG), *P. gingivalis* LPS. Cells were harvested and luciferase activities were measured with a dual-luciferase system. The fold activation was determined by comparing with the basal luciferase activity when cells were cotransfected with 2× κB luciferase reporter and empty vector. The assays were performed in duplicate, and the activation value represents the mean values ± SD from three independent experiments. *, $p < 0.01$. *B*, Overexpression of IKKγC417R inhibited IL-8 expression induced by LPS. Both THP/V and THP/IKKγC417R were treated with LPS for 30 min. Cells were harvested, and total RNA was extracted with TRIzol reagents. Aliquots (15 μg) of total RNA were resolved on a 1.4% agarose-formaldehyde gel and probed with ^{32}P-labeled IL-8 cDNA probes.

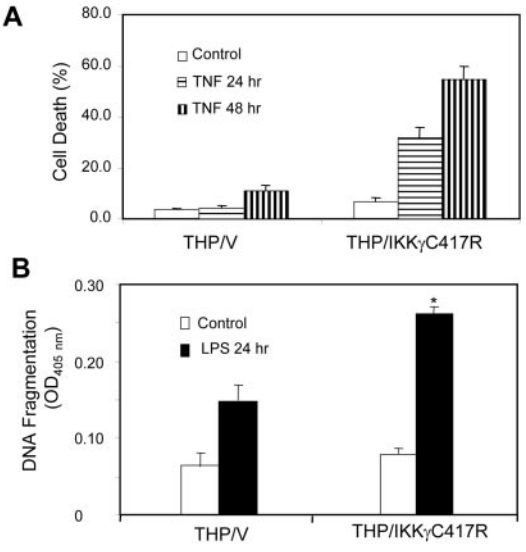

FIGURE 5. Inhibition of NF-κB by IKKγC417R renders cells sensitive to TNF- and LPS-induced apoptosis. *A*, Overexpression of IKKγC417R potentiated TNF killing. Both THP/V and THP/IKKγC417R cells were treated with TNF (20 ng/ml) for the indicated time periods. Cell viability was determined with 0.1% trypan blue. The assays were performed in duplicate, and the percentage of cell death represents the mean values ± SD from three independent experiments. *B*, Overexpression of IKKγC417R enhanced LPS-mediated apoptosis. Cells were treated with LPS for 24 h, and supernatants were collected. Aliquots (20 μl) of supernatants were measured by cell death ELISA (Roche, Basel, Switzerland). The color reaction was measured with a plate reader by determining the OD at the wavelength of 405 nm. The assay was performed in duplicate, and the result represents one of three independent experiments. Statistical significance was determined by the Student's *t* test. *, $p < 0.01$.

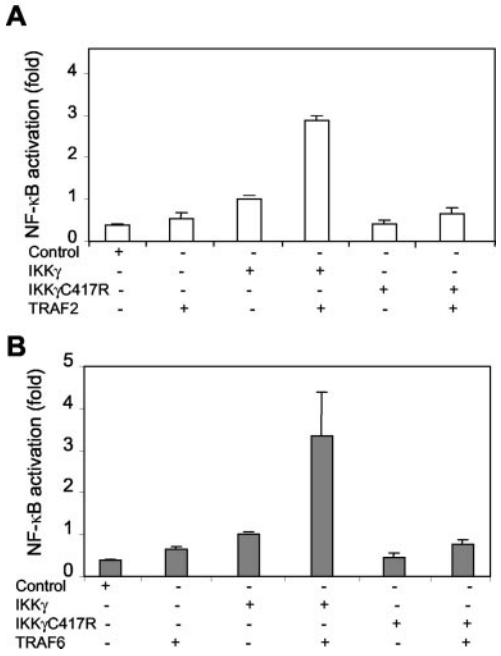

FIGURE 6. The zinc finger structure of IKKγ is essential for LPS- and TNF-induced NF-κB activation. *A*, The zinc finger structure of IKKγ was essential for TRAF2-induced NF-κB transcription. Cells were cotransfected with NF-κB luciferase reporter with TRAF2, IKKγ, or IKKγC417R, as described. Twenty-four hours after transfection, cells were harvested and lyesd. The luciferase activities were measured with a dual luciferase assay system. The assay was performed in triplicate, and the results represent average value from two independent experiments. *B*, The zing finger structure of IKKγ is essential for TRAF6-induced NF-κB transcription. Both cell transfection and luciferase assay were performed, as described in *A*.

that inhibition of NF-κB activation by IKKγC417R increases cell sensitivity to TNF- and LPS-mediated apoptosis.

The zinc finger structure of IKKγ was essential for IKK activation and the nuclear translocation of NF-κB

Recently, Harhaj et al. (30) isolated a mutant T cell line (JM4.5.2) from parental Jurkat T cells that lacked expression of IKKγ using somatic cell mutagenesis. JM4.5.2 cells had been shown to be defective in NF-κB activation in response to a variety of stimuli, including PMA/ionomycin and retroviral oncoprotein Tax. Expression of exogenous IKKγ in JM4.5.2 cells was able to restore NF-κB activation by Tax. Therefore, to further rule out the nonspecific role of overexpression of IKKγ, we were interested in using these mutant cells to determine whether the zinc finger structure of IKKγ played an essential role in LPS- and TNF-mediated NF-κB activation. Unfortunately, although they can be activated by TNF, Jurkat T cells did not respond to LPS stimulation due to the lack of Toll-like receptors. To overcome this barrier, we took advantage of the functional role of TNFR-associated factor (TRAF) signaling molecules in NF-κB activation. It is known that TNF stimulates NF-κB through TRAF2, while LPS-mediated NF-κB activation is mediated by TRAF6. Therefore, we performed transfection assays to determine whether reconstitution of IKKγ or IKKγC417R in JM4.5.2 cells restored TRAF2- and TRAF6-mediated NF-κB activation. As shown in Fig. 6*A*, consistent with the essential role of IKKγ in IKK activation, overexpression of TRAF2 alone could not stimulate NF-κB transcription in JM4.5.2 cells. Coexpression of TRAF2 with IKKγ, but not IKKγC417R, potently activated NF-κB in JM4.5.2 cells. Similarly, while overexpression of TRAF6 alone could not activate NF-κB, coexpression of TRAF6 with IKKγ, but not IKKγC417R, strongly stimulated NF-κB activation in JM4.5.2 cells (Fig. 6*B*). Taken together, our reconstitution experiments demonstrate that the zinc finger structure of IKKγ plays an essential role in LPS- and TNF-induced NF-κB activation.

IKKγC417R inhibited phosphorylation in the activation loop of IKKβ

Biochemical analysis has found that proinflammatory stimuli induced the phosphorylation of two sites (the serines 177 and 181) at the activation loop of IKKβ, resulting in conformational changes and activation of IKK. The mutation of the serines 177 and 181 abolished proinflammatory stimuli-induced IKK activity and NF-κB activation, which suggests that the phosphorylation of the activation loop of IKKβ plays an essential role in IKK activation (31). To further explore the molecular mechanisms by which IKKγC417R inhibited LPS- and TNF-induced IKK activation, we were interested in knowing whether IKKγC417R interfered with the phosphorylation of the activation loop of IKKβ induced by LPS or TNF. As shown in Fig. 7*A*, LPS induced the phosphorylation of the activation loop of IKKβ in THP/V cells or THP/IKKγ cells in a time-dependent manner. In contrast, the phosphorylation of the activation loop of IKKβ induced by LPS was not detected in THP/IKKγC417R cells. Similarly, TNF could not induce the phosphorylation of the activation loop of IKKβ in THP/IKKγC417R cells, whereas TNF stimulated the phosphorylation of the activation loop of IKKβ in THP/V or THP/IKKγ cells (Fig. 7*B*). In summary, our results suggest that IKKγC417R suppresses IKK activation through inhibiting the phosphorylation of the activation loop of IKKβ.

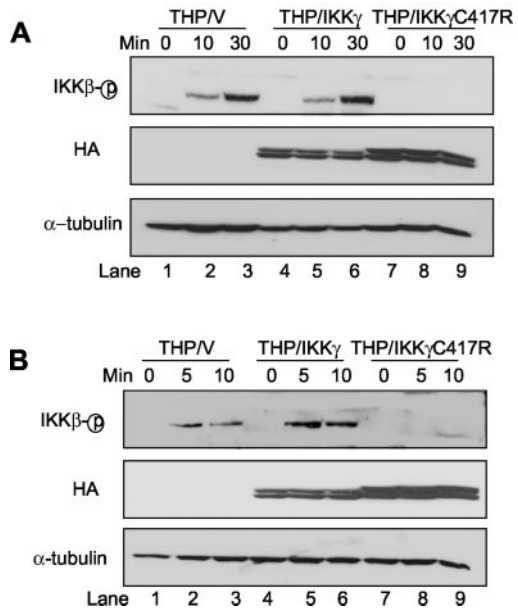

FIGURE 7. The zinc finger mutation in IKKγ inhibited the phosphorylation of the activation loop of IKKβ induced by TNF and LPS. *A*, IKKγC417R inhibited LPS-induced phosphorylation of the activation loop of IKKβ. THP/V, THP/IKKγ, and THP/IKKγC415R cells were treated with *P. gingivalis* LPS (500 ng/ml) for the indicated time periods. Aliquots (50 μg) of whole cell extracts were probed with anti-phospho-IKKβ Abs (1/1000). For loading control, the membranes were stripped and reprobed with anti-HA or anti-α-tubulin. *B*, IKKγC417R inhibited TNF-induced phosphorylation of the activation loop of IKKβ. Cells were treated with TNF (20 ng/ml) for the indicated time periods. Aliquots (50 μg) of whole cell extracts were probed with anti-phospho-IKKβ Abs. For loading control, the membranes were stripped and reprobed with anti-HA or anti-tubulin.

Discussion

IKKγ encodes a regulatory component of the IKK complex in the NF-κB signaling pathway (4, 10–12). Genetic analysis has found that loss-of-function mutations of the *Ikk*γ gene are responsible for familial IP, an X-linked dominant disorder with a variety of developmental abnormalities of skin, teeth, hair, eyes, and CNS in heterozygous females, and death in male fetuses (14). Recently, it has been reported that mutations in the C-terminal region of *Ikk*γ result in ectodermal dysplasia with immunodeficiency and other syndromes (15, 16). Specifically, it is intriguing that the C417 missense mutation in the putative zinc finger structure of IKKγ was identified in patients by several groups (15, 16). Given the fact that C417 is a key residue of the zinc finger structure of IKKγ, in this study, we systemically characterized the effects of the C417R mutation in IKKγ on NF-κB signaling in human immune cells. We found that IKKγC417R significantly inhibited both TNF- and LPS-induced IKK-mediated I-κBα phosphorylation and degradation in a dominant-negative fashion. Consistently, the nuclear translocation and transcription of NF-κB were inhibited by overexpression of IKKγ. Our reconstitution experiments have demonstrated that the zinc finger structure is critical for LPS- and TNF-mediated NF-κB activation. Because of the loss of NF-κB function, cells expressing IKKγC417R were sensitive to TNF- and LPS-induced apoptosis. The EDA-ID or XHM-ED patients' immunodeficiency is associated with NF-κB defect in response to LPS and proinflammatory cytokines.

In contrast to our works, studies by Jain et al. (16) claimed that the C417R mutation in IKKγ has no effect on LPS- and TNF-induced NF-κB activation in cells isolated from patients with XHM-ED. They found that mutations specifically prevented CD40L-mediated degradation of I-κBα. However, it should be pointed out that the experiments for the determination of NF-κB defect were incomplete and problematic in their studies. To examine the effect of the C417R mutation on LPS- and TNF-mediated activation, they only showed data on I-κBα degradation induced by LPS and TNF. Neither IKK activation (I-κBα phosphorylation) nor the nuclear translocation of NF-κB activation was examined and compared. For unknown reasons, to induce I-κBα degradation, they stimulated cells with TNF or LPS in combination with cycloheximide. However, no controls were provided regarding whether cycloheximide treatment alone affected I-κBα degradation. Paradoxically, they only demonstrated that IKKγC417R abolished CD40L-induced I-κBα phosphorylation and did not provide data regarding CD40L-induced I-κBα degradation. Additionally, although they claimed that immunity was preserved in XHM-ED patients, we also noticed that TNF secretion by monocytes was significantly decreased (>40–50%) following LPS plus IFN-γ stimulation in their studies. Because it is well known that TNF expression is regulated by NF-κB, these results suggest that IKKγC417R may have effects on NF-κB activation stimulated by LPS.

In this study, we have extensively examined the IKKγC417R mutant on IKK activation and NF-κB transcription induced by LPS and TNF. Our reconstitution experiments demonstrated that the zinc finger structure of IKKγ plays an essential role in TNF-induced NF-κB activation. Importantly, we found that LPS-induced IL-8 expression was significantly reduced in human THP.1 monocytes by overexpression of IKKγC4177R. It is known that patients with the IKKγC417R mutation have severe bacterial infections. Our results suggest that monocytes are probably defective in producing chemokines to fight against bacterial infection. We and others have previously demonstrated that NF-κB is an important cell survival factor to inhibit apoptosis induced by a variety of apoptotic stimuli. Consistently, we found that IKKγC417R increased cell sensitivity to TNF- and LPS-mediated apoptosis. Our preliminary studies from the microarray analysis found that expression of NF-κB-dependent survival genes induced by LPS was suppressed in human THP.1 monocytes by overexpression of IKKγC417R (our unpublished observation). These results suggest that both an immune response defect and a compromised life span of monocytes may contribute to the immunodeficiency of EDA-IA patients.

Supporting our studies, Markris et al. (32) reported that C417R mutation in IKKγ abolished TNF-induced NF-κB activation in human fibroblasts during the preparation of our manuscript. They also demonstrated that the zinc finger mutation had no effects on IKK complex formation in vivo. Consistent with their studies, we also found that the zinc finger mutation did not interfere with IKK complex formation (our unpublished observation). Interestingly, our new results in this study demonstrated that C417R mutation in IKKγ abolished both TNF- and LPS-induced phosphorylation of the activation loop of IKKβ. Because the phosphorylation of the activation loop of IKKβ is essential for IKK activation (31), our findings provide a molecular explanation for IKKγC417R-mediated inhibition of IKK activation. Finally, given the fact that NF-κB plays an important role in chronic inflammatory diseases such as arthritis, multiple approaches including human gene therapy are being developed to inhibit NF-κB signaling. Our results suggest that the zinc finger structure of IKKγ may be an alternative target for inhibition of inflammation by modifying NF-κB activation.

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