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Suppression of the TRIF-dependent Signaling Pathway of Toll-like Receptor by Cadmium in RAW264.7 Macrophages

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Abstract

Toll-like receptors (TLRs) play an important role in host defense by sensing invading microbial pathogens. The stimulation of TLRs by microbial components triggers the activation of the myeloid differential factor 88 (MyD88)- and toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent downstream signaling pathways. TLR/MyD88 signaling pathway induces the activation of nuclear factor-kappa B (NF-κB) and the expression of inflammatory cytokine genes, including tumor necrosis factor-alpha, interleukin (IL)-6, IL-12, and IL-1β. On the other hand, TLR/TRIF signaling pathway induces the delayed-activation of NF-κB and interferon regulatory factor 3 (IRF3), and the expression of type I interferons (IFNs) and IFN-inducible genes. The divalent heavy metal cadmium (Cd) is clearly toxic to most mammalian organ systems, especially the immune system. Yet, the underlying toxic mechanism(s) remain unclear. Cd inhibits the MyD88-dependent pathway by ceasing the activity of inhibitor-κB kinase. However, it is not known whether Cd inhibits the TRIF-dependent pathway. Presently, Cd inhibited NF-κB and IRF3 activation induced by lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid. Cd inhibited LPS-induced IRF3 phosphorylation and IFN-inducible genes such as interferon inducible protein-10 and regulated on activation normal T-cell expressed and secreted (RANTES). These results suggest that Cd can modulate TRIF-dependent signaling pathways of TLRs.

Keywords: Toll-like receptors, Cadmium Lipopolysaccharide, Polyinosinic-polycytidylic acid, TRIF

Toll-like receptors (TLRs) act as innate immune sensors by recognizing diverse molecular products derived from all the major classes of microbes including bacteria, viruses, yeast, and fungi[1,2]. The activation of these receptors by their respective ligands recruits the two major toll/interleukin-1 receptor (TIR) domain-containing adapter molecules myeloid differentiation protein-88 (MyD88) and TIR domain-containing adapter inducing interferon-β (TRIF).

MyD88 is the immediate adaptor molecule for signaling by all mammalian TLRs except for TLR3[3]. MyD88 recruits interleukin-1 receptor-associated kinase-4 (IRAK-4) to TLRs and phosphorylates IRAK-4. Phosphorylated IRAK-4 induces phosphorylation of IRAK-1 that, in turn, associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), leading to the activation of the canonical IKK complex followed by the activation of the transcription factor nuclear factor-κB (NF-κB). This TLR/MyD88 signaling pathway, which is also called the MyD88-dependent pathway, induces the expression of inflammatory cytokine genes including TNF-α, interleukin (IL)-6, IL-12, and IL-1β[4].

TRIF is an essential adapter molecule for the TLR3- and TLR4-mediated MyD88-independent pathway[4]. TRIF activates two noncanonical IKKs, inducible IKK (IKK*i*)/IKKε and TRAF family member associated NF-κB activator-binding kinase1 (TBK1)/NF-κB-activating kinase (NAK)/TRAF2-associated kinase (T2K), leading to the phosphorylation and activation of interferon regulatory factor (IRF3) and the consequent expression of type I interferons (IFNs) and IFN-inducible genes[5]. IFN and IFN-inducible genes such as interferon inducible protein-10 (IP-10) and regulated on activation normal T-cell expressed and secreted (RANTES) are critical genes regulated by TRIF-dependent signaling pathway of TLRs[2,6,7]. The activation of the TRIF pathway also leads to the activation of NF-κB mediated through RIP1[3].

Cadmium [Cd(II); Cd], a highly ubiquitous heavy metal, is a known occupational hazard and air pollutant[8]. The health effects of Cd have been extensively studied globally. Cd is toxic to most mammalian organ systems, especially the immune system[9]. Chronic exposure to Cd may increase the risk of cerebrovascular

mortality and stroke[10]. Cd causes kidney damage and bone fracture[11,12], and the metal has been linked to kidney cancer[13,14]. Cd increases the blood-brain barrier permeability, resulting in severe dysfunction of the central nervous system[15]. The toxicity of Cd is believed to be largely due to its high affinity for sulfhydryl groups[16].

The effect of Cd on NF-κB activity through the MyD88-dependent pathway of TLRs appears to be mediated through its activation on IKK activity and

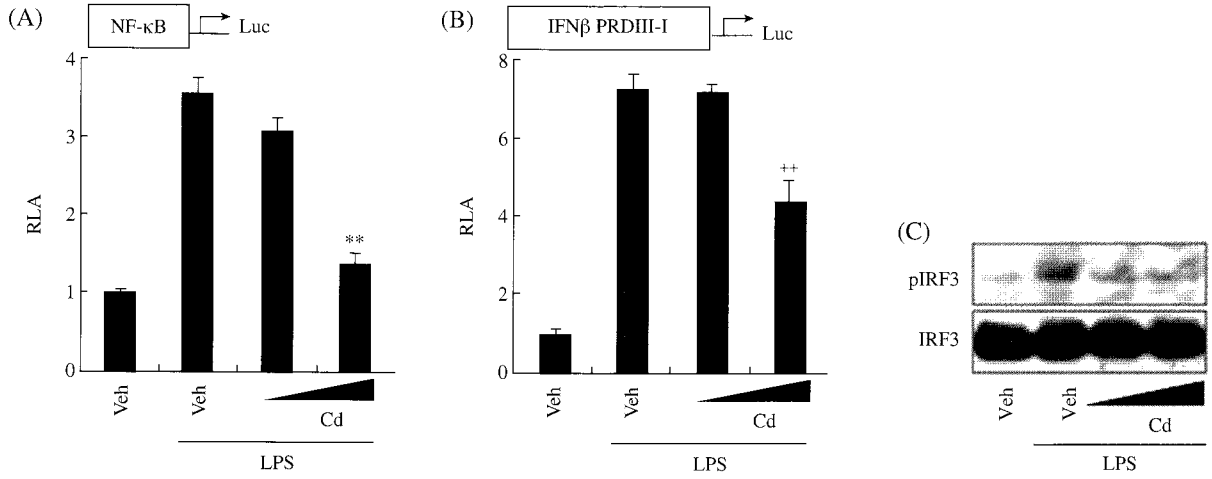

Figure 1. Cadmium inhibited LPS-induced IRF3 activation and phosphorylation. (A, B) RAW264.7 cells were transfected with NF-κB (A) or IRF3 binding site (IFNβ PRDIII-I) (B) luciferase reporter plasmid and pre-treated with cadmium (10, 30 μM) for 1 h and then treated with LPS (10 ng/mL) for an additional 8 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described in Materials and Methods. Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean±SEM (n=3). **, Significantly different from LPS alone (A), $P<0.01$. $^{++}$, Significantly different from LPS alone (B), $P<0.01$. (C) RAW264.7 cells were pretreated with cadmium (10, 30 μM) for 1 h and then further stimulated with LPS (10 ng/mL) for 2 h. Cell lysates were analyzed for phospho-IRF3 (S396) and IRF3 proteins by Western blotting. Veh, vehicle; Cd, cadmium.

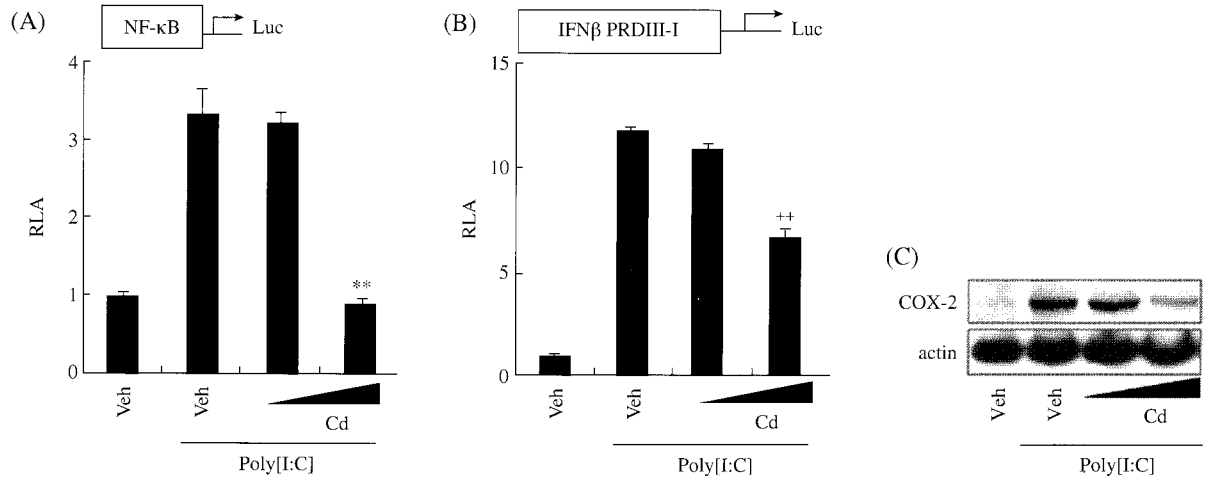

Figure 2. Cadmium inhibited poly[I:C]-induced NF-κB and IRF3 activation. (A, B) RAW264.7 cells were transfected with NF-κB (A) or IRF3 binding site (IFNβ PRDIII-I) (B) luciferase reporter plasmid and pre-treated with cadmium (10, 30 μM) for 1 h and then treated with poly[I:C] (10 μg/mL) for an additional 8 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described in Materials and Methods. Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean±SEM (n=3). **, Significantly different from poly[I:C] alone (A), $P<0.01$. $^{++}$, Significantly different from poly[I:C] alone (B), $P<0.01$. (C) RAW264.7 cells were pretreated with cadmium (10, 30 μM) for 1 hr and then further stimulated with poly[I:C] (10 μg/mL) for 8 hrs. Cell lysates were analyzed for COX-2 and actin protein by immunoblots. Veh, vehicle; Cd, cadmium.

phosphorylation of IκB and NF-κB proteins. However, it is not known if Cd can regulate the TRIF-dependent signaling pathway following TLR activation. Therefore, the present study was designed to explore the effects of Cd on TRIF-dependent signaling pathway of TLRs.

Cadmium Suppresses Lipopolysaccharide (LPS)-induced NF-κB and IRF3 Activation

LPS-induced activation of TLR4 can in turn induce the activation of NF-κB mediated through both MyD88- and TRIF-dependent pathways. Hence, NF-κB activation was used as the readout for LPS-induced TLR4 activation. Cd inhibited NF-κB activation induced by LPS in RAW264.7 cells as determined by the luciferase reporter gene assay (Figure 1A).

Next, we determined whether Cd could inhibit the TRIF-dependent signaling pathway of TLR4. TRIF activates downstream kinases such as TBK1 and IKKi, leading to the phosphorylation and activation of IRF3[5]. Therefore, IRF3 activation was used as the readout for TRIF-dependent pathway. Cd inhibited LPS-induced IRF3 activation as determined by a reporter gene assay using the IFNβ promoter domain containing the IRF3 binding site (IFNβ PRDIII-I) (Figure 1B). Cd also inhibited the phosphorylation of IRF3 as determined by Western blotting (Figure 1C). These results suggest that Cd inhibits the TRIF-dependent signaling pathway derived from TLR4 activation.

Cadmium Suppresses Polyinosinic-polycytidylic acid (Poly[I:C])-induced NF-κB and IRF3 Activation

Although the TLR4 agonist LPS can trigger NF-κB and IRF3 activation mediated through both MyD88- and TRIF-dependent pathways, the TLR3 agonist poly[I:C] triggers NF-κB and IRF3 activation only through the TRIF-dependent pathway. Therefore, NF-κB and IRF3 activation induced by poly[I:C] can be used as the readout for the TRIF-dependent pathway. Cd inhibited poly[I:C]-induced NF-κB and IRF3 activation as determined by a luciferase reporter gene

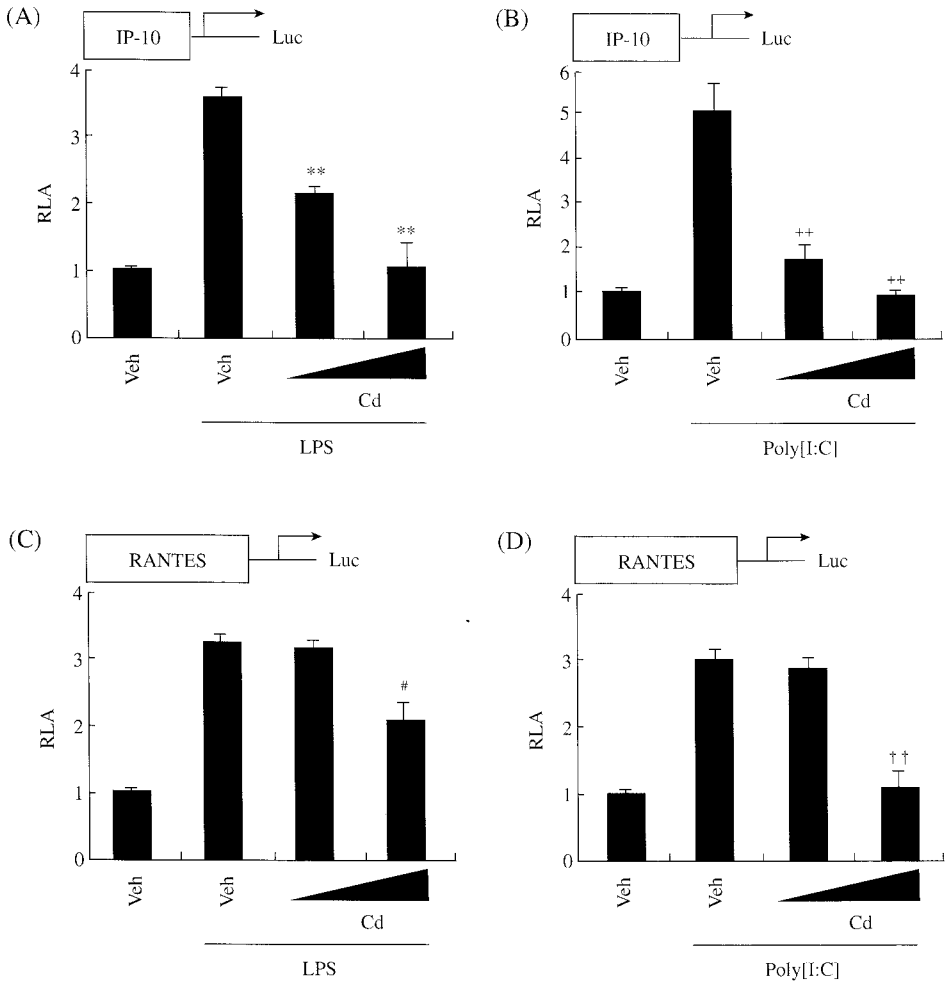

Figure 3. Cadmium inhibited LPS or poly[I:C]-induced IP-10 and RANTES activation. (A-D) RAW264.7 cells were transfected with IP-10 (A, B) or RANTES (C, D) luciferase reporter plasmid and pre-treated with cadmium (10, 30 μM) for 1 h and then treated with LPS (10 ng/mL) (A, C) or poly[I:C] (10 μg/mL) (B, D) for an additional 8 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described in Materials and Methods. Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean ±SEM (n=3). **, Significantly different from LPS alone (A), $P<0.01$. ++, Significantly different from poly[I:C] alone (B), $P<0.01$. #, Significantly different from LPS alone (C), $P<0.05$. ††, Significantly different from poly [I:C] alone (D), $P<0.01$.

assay (Figure 2A and 2B). Cd also inhibited poly[I:C]-induced cyclooxygenase-2 (COX-2) expression as determined by Western blotting (Figure 2C).

Cd Suppresses LPS or Poly[I:C]-induced IP-10 and RANTES Expression

To further investigate Cd regulation of the TRIF-dependent pathway, expression of genes associated with the TRIF-dependent pathways such as IP-10 and RANTES were measured by the luciferase reporter gene assay. Cd inhibited IP-10 and RANTES expression induced by LPS and poly[I:C] (Figure 3), consistent with the suggestion that Cd inhibits TRIF-dependent signaling pathway of TLRs for the inhibition of NF-κB and IRF3.

Discussion

All TLR signaling pathways culminate in NF-κB activation. The activation of NF-κB induced by microbial pathogens requires phosphorylation and subsequent degradation with a family of the inhibitor proteins known as IκB through the canonical IKK complex composed of two catalytic kinase subunits, IKKα and IKKβ[17-19]. Although LPS signaling through TLR4 utilizes both the MyD88- and TRIF-dependent pathways, TLR3 signals occur only via the TRIF pathway[3]. Activation of the intracellular MyD88- and TRIF-dependent pathway leads to time-dependent differences in activation of NF-κB; activation of the MyD88-dependent pathway results in early activation of NF-κB, whereas activation of the TRIF-dependent pathway results in delayed activation of NF-κB.

In addition to NF-κB activation, recognition of viral double-stranded RNA by TLR3 and bacterial LPS by TLR4 leads to IRF3 activation mediated through the TRIF-dependent signaling pathway[20,21]. Activation of TLR3 and TLR4 recruits TRIF that activates the downstream kinases, TBK1 (TANK-binding kinase1) and IKKε (inhibitor-κB kinase-ε), leading to IRF3 activation[5]. Activated IRF3 becomes readily phosphorylated, resulting in IRF3 dimerization and subsequent translocation to the nucleus. The translocated IRF3 binds to consensus DNA sequences known as interferon (IFN)-stimulated response element found in the promoter regions of genes such as those encoding IFN-β and RANTES[22,23]. These IRF3-regulated genes play an important role in both the anti-viral and anti-bacterial innate immune responses[24]. Since the TRIF-dependent pathway is responsible for the expression of more than 70% of LPS-inducible genes[25], modulation of the TRIF-dependent pathway of TLRs might be a novel anti-inflammatory strategy. Our results show that Cd suppresses IFNβ production induced by TLR3 or TLR4 agonists and the transcriptional activation of IRF3 by inhibiting TRIF-dependent signaling pathway.

Cd, a highly ubiquitous heavy metal, is known to cause inflammation in various tissues, which are important processes operating in Cd-induced pathological conditions[26]. Cd has a high affinity for sulfhydryl groups[27]. Its cellular toxicity is through direct interaction with thiol groups of proteins. A thiol-based mechanism for Cd inhibition of protein-DNA interactions has been demonstrated in transcription factor IIIA-DNA binding studies, in which the binding site of Cd was proposed to be the Cys_2Cys_2 zinc finger domain[28]. Other studies have shown that Cd inhibits the activities of glutathione peroxidase, catalase, and superoxide dismutase through the binding of sulfur atoms or the sulfur analog selenium[27,29]. Inhibition of NF-κB-DNA binding by Cd appears to be the result of metal-mediated targeting of thiols[30].

In a previous report, we showed that Cd inhibits NF-κB activation and COX-2 expression induced by TLR2 and TLR4 agonists[31]. In addition, we presently demonstrate for the first time that Cd suppresses TRIF-dependent pathway of TLRs. This suppression is accompanied by the down-regulation of the activation of NF-κB and IRF3, and of their target genes including IFNβ, IP-10, and RANTES. These results further the understanding of the mechanism of modulation of TLRs by Cd.

Materials & Methods

Reagents

Cadmium chloride ($CdCl_2$) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in dimethyl sulfoxide. Purified LPS and poly[I:C] were purchased from List Biological Lab. (San Jose, CA, USA) and Amersham Biosciences (Piscataway, NJ, USA), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise described.

Cell Culture

RAW 264.7 cells (a murine monocytic cell line, ATCC TIB-71) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL Penicillin, and 100 μg/mL Streptomycin (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C in a 5% CO_2/air environment.

Plasmids

NF-κB (2x)-luciferase reporter construct was pro-

vided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA, USA). Heat shock protein 70 (HSP70)-β-galactosidase reporter plasmid was from Robert Modlin (University of California at Los Angeles). IP-10- and RANTES-luciferase reporter constructs were from Dr. Daniel Hwang (University of California at Davis). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA, USA) for transfection.

Transfection and Luciferase Assays

Transfection and luciferase assays were performed as described previously[32,33]. Briefly, RAW264.7 cells were co-transfected with a luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen) according to the manufacturer's instructions. Luciferase and β-galactosidase enzyme activities were determined using the luciferase assay system and β-galactosidase enzyme system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was normalized to β-galactosidase activity. Data was obtained from triplicate experiments. Values were expressed as mean ± standard error mean (SEM).

Western Blotting

Western blotting was performed as previously described[34,35]. In each experiment, equal amounts of extracts were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked with phosphate-buffered saline containing 0.1% Tween-20 and 3% nonfat dry milk, and were blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ, USA). The reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences).

References

1. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A., Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**:394-397 (1997).
2. Bjorkbacka, H. *et al.* The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. *Physiol Genomics* **19**: 319-330 (2004).
3. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int Immunol* **17**:1-14 (2005).
4. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**:783-801 (2006).
5. Fitzgerald, K. A. *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* **4**:491-496 (2003).
6. Kawai, T. *et al.* Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* **167**:5887-5894 (2001).
7. Gao, J. J. *et al.* Autocrine/paracrine IFN-alphabeta mediates the lipopolysaccharide-induced activation of transcription factor Stat1alpha in mouse macrophages: pivotal role of Stat1alpha in induction of the inducible nitric oxide synthase gene. *J Immunol* **161**:4803-4810 (1998).
8. Jarup, L. Hazards of heavy metal contamination. *Br Med Bull* **68**:167-182 (2003).
9. Christensen, M. M., Ellermann-Eriksen, S., Rungby, J. & Mogensen, S. C. Influence of mercuric chloride on resistance to generalized infection with herpes simplex virus type 2 in mice. *Toxicology* **114**:57-66 (1996).
10. Elliott, P. *et al.* Risk of mortality, cancer incidence, and stroke in a population potentially exposed to cadmium. *Occup Environ Med* **57**:94-97 (2000).
11. Buchet, J. P. *et al.* Renal effects of cadmium body burden of the general population. *Lancet* **336**:699-702 (1990).
12. Alfven, T. *et al.* Low-level cadmium exposure and osteoporosis. *J Bone Miner Res* **15**:1579-1586 (2000).
13. Kolonel, L. N. Association of cadmium with renal cancer. *Cancer* **37**:1782-1787 (1976).
14. Mandel, J. S. *et al.* International renal-cell cancer study. IV. Occupation. *Int J Cancer* **61**:601-605 (1995).
15. Shukla, A., Shukla, G. S. & Srimal, R. C. Cadmium-induced alterations in blood-brain barrier permeability and its possible correlation with decreased microvessel antioxidant potential in rat. *Hum Exp Toxicol* **15**: 400-405 (1996).
16. Zalups, R. K. & Ahmad, S. Molecular handling of cadmium in transporting epithelia. *Toxicol Appl Pharmacol* **186**:163-188 (2003).
17. Kawai, T. & Akira, S. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med* **13**:460-469 (2007).
18. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M. & Karin, M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* **91**:243-252 (1997).
19. Yamaoka, S. *et al.* Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell* **93**:1231-1240 (1998).
20. Lin, R., Heylbroeck, C., Pitha, P. M. & Hiscott, J. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol Cell Biol* **18**:2986-2996 (1998).
21. Navarro, L. & David, M. p38-dependent activation of interferon regulatory factor 3 by lipopolysaccharide. *J*

Biol Chem **274**:35535-35538 (1999).
22. Lin, R., Heylbroeck, C., Genin, P., Pitha, P. M. & Hiscott, J. Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription. *Mol Cell Biol* **19**:959-966 (1999).
23. Schafer, S. L., Lin, R., Moore, P. A., Hiscott, J. & Pitha, P. M. Regulation of type I interferon gene expression by interferon regulatory factor-3. *J Biol Chem* **273**:2714-2720 (1998).
24. Honda, K. & Taniguchi, T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* **6**:644-658 (2006).
25. Bjorkbacka, H. *et al.* The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. *Physiol Genomics* **19**:319-330 (2004).
26. Manca, D., Ricard, A. C., Tra, H. V. & Chevalier, G. Relation between lipid peroxidation and inflammation in the pulmonary toxicity of cadmium. *Arch Toxicol* **68**:364-369 (1994).
27. Stohs, S. J. & Bagchi, D. Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med* **18**: 321-336 (1995).
28. Hanas, J. S. & Gunn, C. G. Inhibition of transcription factor IIIA-DNA interactions by xenobiotic metal ions. *Nucleic Acids Res* **24**:924-930 (1996).
29. Splittgerber, A. G. & Tappel, A. L. Inhibition of glutathione peroxidase by cadmium and other metal ions. *Arch Biochem Biophys* **197**:534-542 (1979).
30. Shumilla, J. A., Wetterhahn, K. E. & Barchowsky, A. Inhibition of NF-kappa B binding to DNA by chromium, cadmium, mercury, zinc, and arsenite in vitro: evidence of a thiol mechanism. *Arch Biochem Biophys* **349**:356-362 (1998).
31. Ahn, S. I., Park, S. K., Lee, M. Y. & Youn, H. S. Cadmium but not mercury suppresses NF-kB activation and COX-2 expression induced by Toll-like receptor 2 and 4 agonists. *Mol Cell Toxicol* **5**:141-146 (2009).
32. Youn, H. S., Ahn, S. I. & Lee, B. Y. Guggulsterone suppresses the activation of transcription factor IRF3 induced by TLR3 or TLR4 agonists. *Int Immunopharmacol* **9**:108-112 (2009).
33. Ahn, S. I., Lee, J. K. & Youn, H. S. Inhibition of homodimerization of toll-like receptor 4 by 6-shogaol. *Mol Cells* **27**:211-215 (2009).
34. Youn, H. S. *et al.* Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. *J Immunol* **175**:3339-3346 (2005).
35. Youn, H. S., Saitoh, S. I., Miyake, K. & Hwang, D. H. Inhibition of homodimerization of Toll-like receptor 4 by curcumin. *Biochem Pharmacol* **72**:62-69 (2006).