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## Auranofin, as an anti-rheumatic gold compound suppresses LPS-induced homodimerization of TLR4

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### Abstract

Toll-like receptors (TLRs), which are activated by invading microorganisms or endogenous molecules, evoke immune and inflammatory responses. TLR activation is closely linked to the development of many chronic inflammatory diseases including rheumatoid arthritis. Auranofin, an Au(I) compound, is a well-known and long-used anti-rheumatic drug. However, the mechanism as to how auranofin relieves the symptom of rheumatoid arthritis has not been fully clarified. Our results demonstrated that auranofin suppressed TLR4-mediated activation of transcription factors, NF- $\kappa$ B and IRF3 and expression of COX-2, a pro-inflammatory enzyme. This suppression was well correlated with the inhibitory effect of auranofin on the homodimerization of TLR4 induced by an agonist. Furthermore, auranofin inhibited NF- $\kappa$  activation induced by MyD88-dependent downstream signaling components of TLR4, MyD88, IKK $\beta$ , and p65. IRF3 activation induced by MyD88-independent signaling components, TRIF and TBK1, was also downregulated by auranofin. Our results first demonstrate that auranofin suppresses the multiple steps in TLR4 signaling, especially the homodimerization of TLR4. The results suggest that the suppression of TLR4 activity by auranofin may be the molecular mechanism through which auranofin exerts anti-rheumatic activity.

### Keywords

auranofin; gold; Toll-like receptor; LPS; dimerization; NF- $\kappa$ B; COX-2; MyD88; TRIF; IKK $\beta$

### 1. Introduction

Toll-like receptors (TLRs) play an important role in innate immune responses that are essential for host defense against invading microbial pathogens [1-4]. TLRs interact with different

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A list of non-standard abbreviations: TLR, Toll-like receptors; LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; NF $\kappa$ B, nuclear factor  $\kappa$ B; IRF-3, IFN-regulatory factor-3; MyD88, myeloid differential factor 88; TRIF, TIR domain-containing adapter inducing IKK, I $\kappa$ B kinase; TBK1, TANK-binding kinase 1; IRAK1, interleukin-1 receptor-associated kinase 1.

combinations of adapter proteins. TLR signaling pathways can trigger the activation of NF- $\kappa$  through the MyD88 dependent pathway, but they also induce type I IFN expression through the MyD88-dependent and -independent pathways. MyD88 is the immediate adaptor molecule which is common to all mammalian TLRs [1]. MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) and induces phosphorylation of IRAK-4 leading to the phosphorylation of IRAK-1. The phosphorylated IRAK-1 associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) leading to the activation of the canonical IKK complex resulting in the activation of NF- $\kappa$  transcription factor. The activation of NF- $\kappa$  leads to the induction of inflammatory gene products such as cytokines and cyclooxygenase-2 [5].

TLR3 and TLR4 activate MyD88-independent signaling pathway mediated through TIR domain-containing adapter inducing IFN $\beta$  (TRIF). TRIF-dependent pathway triggers the expression of type I IFN mediated through TBK1 and IKK $\epsilon$  [6,7]. The C-terminal portion of TRIF also was shown to be associated with RIP1 leading to the delayed activation of NF- $\kappa$  [8]. Thus, TRIF is likely to use TBK1 for IRF3- and RIP1 for NF- $\kappa$  activation [9].

Deregulated activation of TLRs can lead to severe systemic inflammatory and joint destructive process in rheumatoid arthritis (RA) [10]. RA is an autoimmune disease characterized by chronic inflammation in most major joints [10]. There are several anti-rheumatic drugs that can be divided into two groups. First, the non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, are used to suppress pain and inflammation. Second, disease-modifying anti-rheumatic drugs (DMARDs) are used to limit joint damage and to suppress the underlying autoimmune dysfunction of RA [11]. The latter class of drugs includes methotrexate, chloroquine, sulphasalazine, D-penicillamine, and gold compounds such as auranofin. The mechanism for the efficiency of these drugs on RA has not been fully understood. Recent strategies for drug development have focused on cytokines that are detected in the synovium of RA. Many cytokines such as TNF, IL-1, IL-6, IL-8, IL-10, IL-12, IL-15 and IL-18 are detected in the RA except for IL-4 [12,13]. The expression of these inflammatory cytokines can be regulated by TLR-NF- $\kappa$  signaling pathway [10].

Gold compounds have been used for the treatment of RA for more than 100 years [14]. The two most common gold compounds used as anti-rheumatic drugs are sodium aurothiomalate, which is water-soluble and administered by the intramuscular route, and auranofin, which is a hydrophobic compound and can be taken orally [14]. Both compounds are Au(I) compounds with sulfur-linked organic ligands. These gold compounds inhibited endotoxin-induced IL-1 and TNF production in the inflammatory monocytes and macrophages in the RA synovial membranes [15,16]. Gold compounds have also been shown to inhibit the endotoxin-induced activation of NF- $\kappa$ B and AP-1 dependent transfected reporter genes [17,18].

Identifying the direct targets of gold compounds in TLR pathways would be important because the activation of TLRs by agonists can induce inflammatory responses and consequently increases the risk of the development of chronic inflammatory diseases. Therefore, we attempted to identify the molecular target of gold compounds in TLR signaling pathways.

## 2. Materials and Methods

### 2.1. Reagents

Auranofin was purchased from Biomol (Plymouth Meeting, PA). Sodium tetrachloroaurate (III) dihydrate was purchased from Sigma-Aldrich (St. Louis, MO). Purified LPS was obtained from List Biological Lab. Inc. All other reagents were purchased from Sigma unless otherwise described.

## 2.2. Cell culture

Ba/F3 cells, an IL-3-dependent murine pro-B cell line, expressing TLR4 (Flag or GFP-tagged), CD14, MD2 (Flag-tagged), and NF- $\kappa$ B luciferase reporter gene were described previously [19]. Cells were cultured in RPMI1640 medium containing recombinant murine IL-3 (70 U/ml), 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 units/ml Penicillin, and 100  $\mu$ g/ml Streptomycin (GIBCOBRL). RAW264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293T cells (human embryonic kidney) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS, 100 units/ml Penicillin, and 100  $\mu$ g/ml Streptomycin. Cells were maintained at 37°C in a 5% CO<sub>2</sub>/air environment.

## 2.3. Transfection and reporter gene luciferase assay

NF- $\kappa$ B(2x)-luciferase reporter gene assay were performed as described previously [20,21]. Cells were co-transfected with a luciferase plasmid and HSP70- $\beta$ -galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Various expression plasmids or equal amounts of empty vector for signaling components were co-transfected. Luciferase enzyme activities were determined using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by  $\beta$ -galactosidase activity.

## 2.4. Immunoblotting and immunoprecipitation

These were performed the same as previously described [20]. Protein extracts from Ba/F3 cells expressing TLR4 (Flag or GFP-tagged), CD14, MD2 (Flag-tagged), and NF- $\kappa$ B luciferase reporter gene for immunoprecipitation were prepared as described [19]. The samples were immunoprecipitated with mouse-GFP antibody (Molecular Probes Inc., Eugene, OR) for overnight. The solubilized immune complex was resolved on 8% SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membrane was blocked with phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk and was blotted with the Flag antibodies for overnight. Thereafter, the blot was exposed to horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Arlington Heights, IL) for 1 hr, and detected with ECL Western blot detection reagents (Amersham Biosciences, Arlington Heights, IL). The blot was reprobed with rabbit GFP antibodies.

## 3. Results

### 3.1. Auranofin inhibited LPS-induced NF- $\kappa$ B and IRF3 activation and target gene (COX-2) expression

Auranofin, an anti-rheumatic gold compound, inhibited LPS-induced activation of NF- $\kappa$ B and IRF3 as determined by luciferase reporter gene assays in RAW264.7 cells (Fig. 1A,B). The decrease in the activation of these transcription factors resulted in the reduction of the expression of the target gene, COX-2 (Fig. 1C).

### 3.2. LPS-induced dimerization of TLR4 was inhibited by gold compounds

We next determined whether auranofin inhibits LPS-induced dimerization of TLR4 using IL-3-dependent Ba/F3 cells stably transfected with murine TLR4-Flag and TLR4-GFP, MD2, CD14, and NF- $\kappa$ B-luciferase reporter gene as described previously [19,22]. Auranofin inhibited the dimerization of TLR4 (Fig. 2A) induced by LPS in a dose dependent manner. The inhibitory potency of auranofin [Au(I)] is greater than that of another gold compound, tetrachloroauric (III) acid [Au(III)]. The inhibition of TLR4 dimerization by the gold compounds was well correlated the suppressive effects on NF- $\kappa$ B activation (Fig. 2B).

### 3.3. Gold compounds inhibited both MyD88-dependent and TRIF-dependent signaling pathways of TLR4

TLR4 activates both MyD88-dependent and independent pathways. Since the gold compounds inhibit the LPS-induced dimerization of TLR4. They should inhibit both MyD88-dependent and -independent pathways. TLR4 activation induced by LPS leads to the recruitment of MyD88. MyD88 in turn recruits IRAK-4 which phosphorylates IRAK-1 leading to its degradation. IRAK-1 associates with TRAF6 leading to the activation of IKK $\beta$  complex resulting in the activation of NF- $\kappa$ B transcription factor. Both auranofin and Au (III) acid inhibited NF- $\kappa$ B activation induced by the overexpression of MyD88, IKK $\beta$ , or p65 in 293T cells (Fig. 3A-C). LPS-induced IRAK-1 degradation was also inhibited by gold compounds (Fig. 3D).

The results suggest that gold compounds inhibit NF- $\kappa$ B activation induced through MyD88-dependent pathway of TLR4. The TRIF-dependent pathway of TLR4 leads to the activation of IRF3 transcription factor mediated through TBK1 [23]. Gold compounds also inhibited TRIF or TBK1 (downstream kinase of TRIF-dependent pathway)-induced IRF3 activation as demonstrated by the transcription activation and the phosphorylation of IRF3 (Fig. 4A-C). These results showed that Gold compounds inhibit TRIF/TBK1/IRF3 signaling pathway of TLR4.

Together, our results demonstrate that gold compounds have the multiple targets in TLR4 signaling pathways to inhibit the NF- $\kappa$ B and IRF3 activation.

## 4. Discussion

Several lines of evidence have shown the significant role of TLR4 in the development and progress of rheumatoid arthritis. Repeated exposure to LPS, a TLR4 agonist, generates a mouse model of reactive arthritis [24]. The endogenous TLR4 agonists such as fibrinogen and hyaluronan are found in arthritis joints. Small heat shock protein B8 (HSP22) abundantly expressed in synovial tissue from patients with rheumatoid arthritis was identified as a TLR4 agonist [25]. Mice deficient of MyD88, an adaptor molecule of TLR, did not develop streptococcal cell wall-induced arthritis [26]. Some anti-rheumatic gold compounds have been shown to suppress LPS activity. Auranofin and gold sodium thiomalate inhibited LPS-induced production of IL-1 or TNF in peripheral blood mononuclear cells or murine macrophages [15,16]. Therefore, the anti-rheumatic activity of gold compounds has been partly explained by the downregulation of the expression of inflammatory mediators. However, the mechanism through which gold compounds suppress LPS activity has not been fully understood. The homodimer formation of TLR4 in response to an agonist is considered as a critical step to recruit the adaptor molecules and to trigger the activation of downstream signaling pathways [19]. Our study demonstrated that auranofin, an anti-rheumatic gold compound, and tetrachloroauric (III) acid to a lesser extent, suppress LPS-induced formation of TLR4 homodimers. The suppression resulted in the decrease of the activation of downstream transcription factors, IRF3 and NF- $\kappa$ B. Therefore, our results suggest a novel mechanism as to how gold compounds suppress LPS activity and exert anti-rheumatic activity.

The biochemical mechanism as to how auranofin interacts with TLR4 to exert the inhibitory effects is not understood. Cysteine residues have been implicated as potential targets for gold compounds. Gold(I) salts have a high thiol binding affinity. Auranofin inhibits I $\beta$ B kinase but the cysteine mutant of IKK $\beta$  (C179A) is resistant to inhibition [27]. This suggests that auranofin inhibits IKK by modifying Cys<sup>179</sup> of IKK $\beta$ . In addition, gold salts react with Cys<sup>272</sup> and Cys<sup>154</sup> in the DNA-binding domains of Jun and Fos, respectively, thereby inhibiting DNA binding and transcription of AP-1 transcription factor [28]. TLRs have several cysteine residues, which form disulfide bonds for the dimerization of the receptors, in both cytoplasmic

and extracellular domains [29]. Therefore, it can be speculated that auranofin may bind to cysteine residues of TLR4 to interfere with the activation.

The engagement of TLR4 by LPS establishes stably sustained phase of NF- $\kappa$ B activation composed of the early phase that is dependent on MyD88 and the TRIF-dependent delayed phase [30]. The activation of IRF3 through TRIF and TBK1 is responsible for the late phase of NF- $\kappa$ B activation. IRF3 activation leads to the expression of TNF $\alpha$  which in turn acts on TNF $\alpha$  receptor to trigger the delayed activation of NF- $\kappa$ B. Therefore, TRIF/TBK1/IRF3 pathway is now recognized to play an important role in TLR-mediated inflammation. Our results showed that auranofin suppressed the functional activity of TRIF and TBK1 as demonstrated by the phosphorylation and the transcriptional activation of IRF3.

Several gold compounds such as auranofin, aurothiomalate, aurothioglucose, and AuCl<sub>3</sub> were shown to inhibit kinase activity of IKK immune complex prepared by the immunoprecipitation with IKK $\alpha$  antibody [31]. This was suggested as the mechanism through which gold compounds suppress NF- $\kappa$ B activation. Consistently, auranofin, aurothioglucose and aurothiomalate inhibited 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced nuclear translocation of NF- $\kappa$ B in rat peritoneal macrophages when cells were treated with each of the gold compounds [32]. However, the contradicting results on the effect of each gold compound on NF- $\kappa$ B activation were also reported. Aurothiomalate did not suppress TNF $\alpha$ -induced NF- $\kappa$ B activation in HUVEC as determined by EMSA while auranofin inhibited the NF- $\kappa$ B activation [33]. Our study demonstrated that auranofin inhibited IKK $\beta$ -induced NF- $\kappa$ B reporter activity. In addition, auranofin and tetrachloroauric (III) acid suppressed p65-induced NF- $\kappa$ B reporter activity suggesting the direct inhibition of p65 transcriptional activity by gold compounds.

Collectively, our study for the first time demonstrated that auranofin inhibits the dimerization of TLR4 and the activation of TRIF-dependent signaling pathways. Auranofin and other gold compound suppress TLR signaling at multiple steps. These results provide an important clue to understand the mechanism for gold compounds to exert anti-rheumatic activity.

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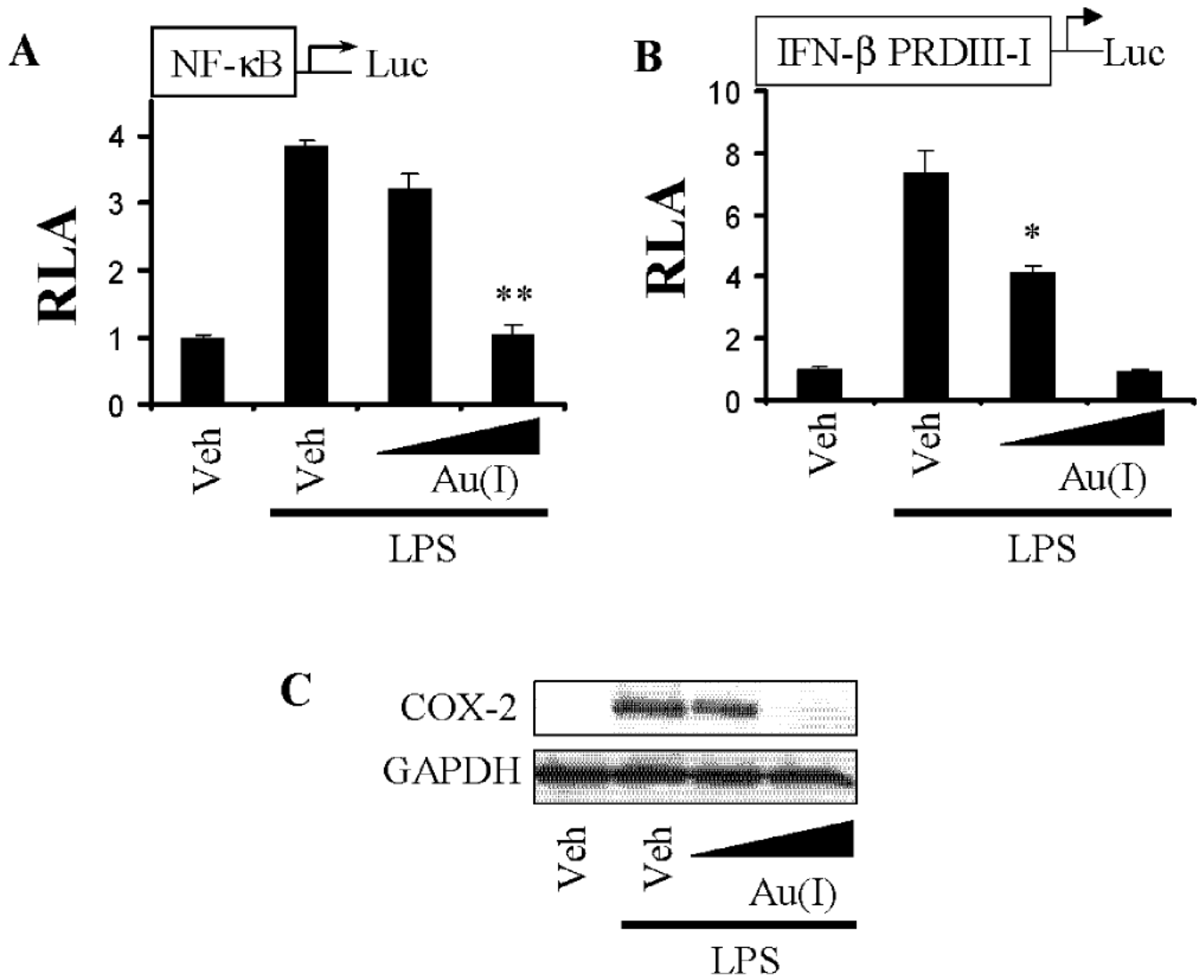
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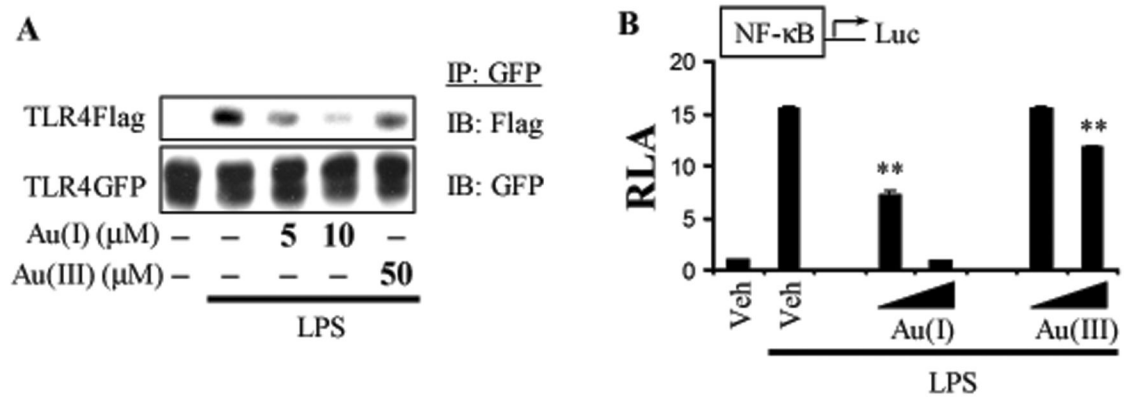
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**Figure 1. Auranofin inhibits LPS-induced NF-κB and IRF3 activation**

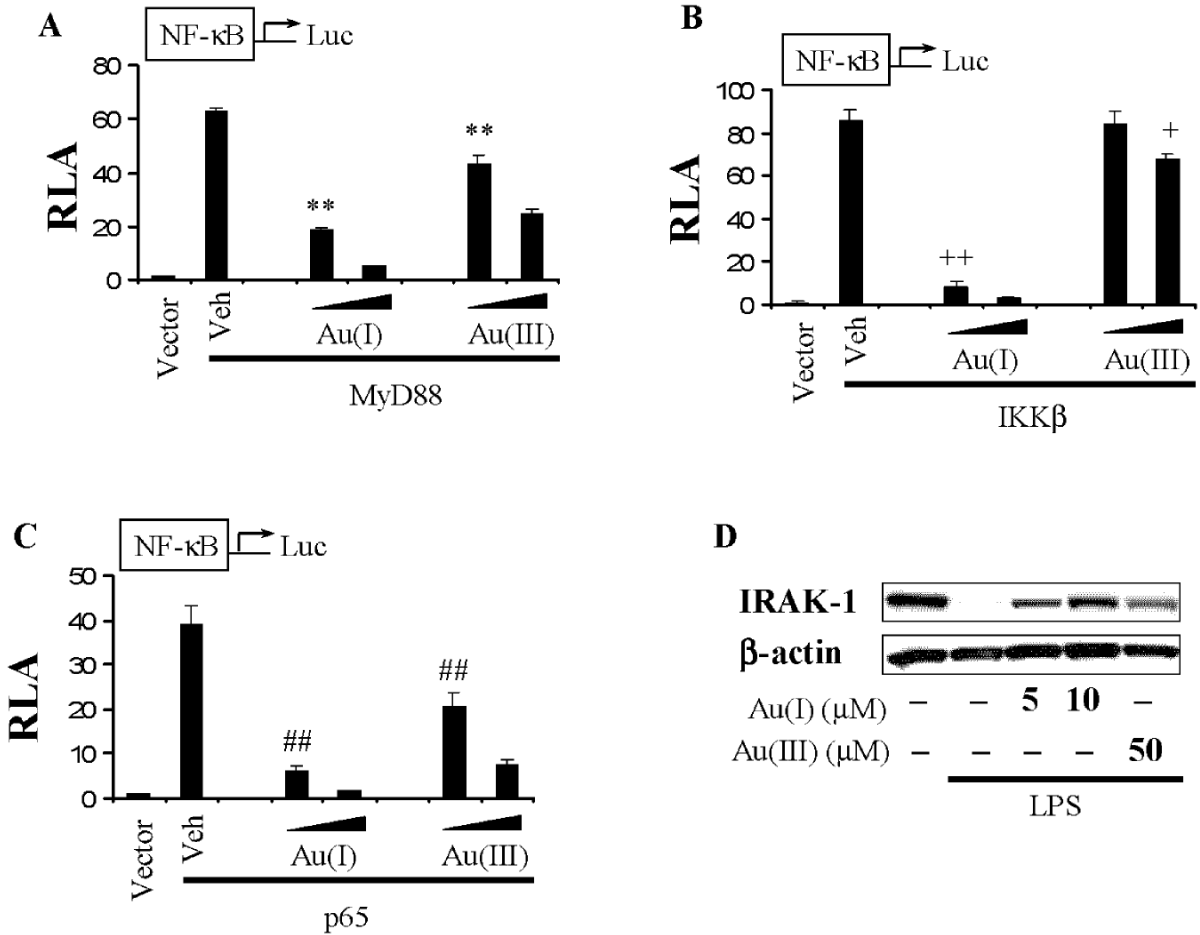
**A,B** RAW264.7 cells were transfected with (A) NF-κB or (B) IFNβ promoter with specific IRF3 binding site (IFNβ PRDIII-I) luciferase reporter plasmid and pre-treated with auranofin (5, 10 μM) for 1 h, and then treated with LPS (5 ng/ml) for an additional 6 hrs. Cell lysates were prepared and luciferase 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,  $p<0.05$ . \*\*, Significantly different from LPS alone,  $p<0.01$ . **C** RAW264.7 cells were pretreated with auranofin (5, 10 μM) for 1 hr and then further stimulated with LPS (5 ng/ml) for 6 hrs. Cell lysates were analyzed for COX-2 and GAPDH protein by immunoblots. The panels are representative data from more than three independent experiments. Veh, vehicle; Au(I), auranofin.





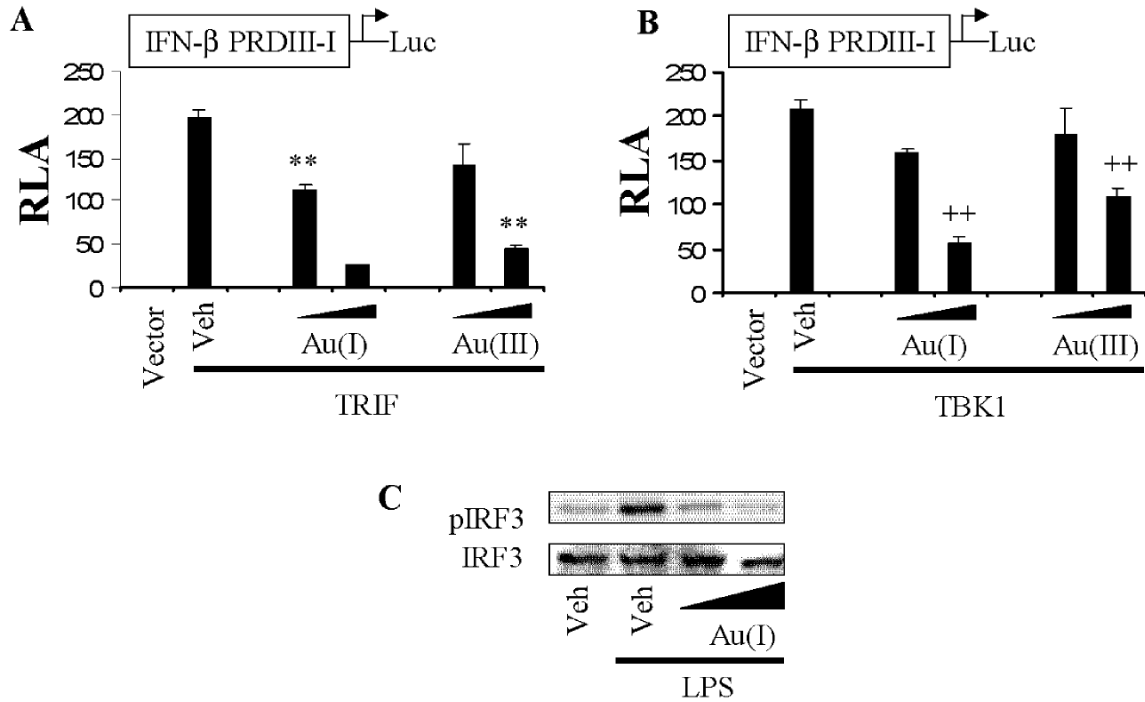
**Figure 2. Gold compounds inhibit LPS-induced homodimerization of TLR4**

**A)** Ba/F3 cells expressing TLR4-Flag (TLR4F), TLR4-GFP (TLR4G), MD2-Flag (MD2F), CD14 and NF- $\kappa$ B luciferase were pre-treated with auranofin (5, 10  $\mu\text{M}$ ) or sodium tetrachloroaurate (50  $\mu\text{M}$ ) for 1 h and then treated with LPS (50 ng/ml) for 20 mins. Cells were then subjected to immunoprecipitation with anti-mouse GFP antibody and immunoblotted with anti-Flag (upper) or anti-rabbit GFP (lower) antibody. **B)** The same Ba/F3 cells in Fig. 2A were pre-treated with auranofin (5, 10  $\mu\text{M}$ ) or sodium tetrachloroaurate (20, 50  $\mu\text{M}$ ) for 1 h and then treated with LPS (5 ng/ml) for an additional 6 hrs. Cell lysates were prepared and luciferase enzyme activities were measured as described in the legend of Fig. 1. Values are mean $\pm$ SEM (n=3). \*\*, Significantly different from LPS alone,  $p < 0.01$ . The panels are representative data from more than three independent experiments. Veh, vehicle; Au(I), auranofin; Au(III), sodium tetrachloroaurate dihydrate.



**Figure 3. Gold compounds inhibit MyD88-dependent signaling pathways**

**A-C** 293T cells were transfected with NF-κB binding site(2x)-luciferase reporter plasmid and the expression plasmid of (A) MyD88, (B) IKKβ, or (C) p65. After 24 hrs, cells were further treated with auranofin (5, 10 μM) or sodium tetrachloroaurate (20, 50 μM) for 6 hrs. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. Values are mean ±SEM (n=3). (A) \*\*, Significantly different from MyD88 plus vehicle,  $p < 0.01$ . (B) ++, Significantly different from IKKβ plus vehicle,  $p < 0.01$ . +, Significantly different from IKKβ plus vehicle,  $p < 0.05$ . (C) ##, Significantly different from p65 plus vehicle,  $p < 0.01$ . **D**) RAW264.7 cells were pretreated with auranofin (5, 10 μM) for 1 h and then stimulated with LPS (50 ng/ml) for 30 mins. Cell lysates were subjected to SDS-PAGE and probed with anti-IRAK-1 (upper) or anti-β-actin (lower) antibody. The panels are representative data from more than three independent experiments. Veh, vehicle; Au(I), auranofin; Au(III), sodium tetrachloroaurate dihydrate.



**Figure 4. Gold compounds inhibit TRIF or TBK1-induced IRF3 activation**

**A, B**) 293T cells were co-transfected with IFN $\beta$  promoter (IFN $\beta$  PRDIII-I)-luciferase reporter plasmid and an expression plasmid for TRIF or TBK1. pCMV for TRIF and pcDNA for TBK1 were used as a vector control. After 24 hrs, cells were treated with auranofin (5, 10  $\mu$ M) or sodium tetrachloroaurate (20, 50  $\mu$ M) for 6 hrs. Relative luciferase activity (RLA) was determined by normalization with  $\beta$ -galactosidase activity. Values are mean $\pm$ SEM (n=3). (A) \*\*, Significantly different from TRIF plus vehicle,  $p < 0.01$ . (B) ++, Significantly different from TBK1 plus vehicle,  $p < 0.01$ . **C**) RAW264.7 cells were treated with auranofin (5, 10  $\mu$ M) for 1 h and further stimulated with LPS (50 ng/ml) for 1.5 h. Cell lysates were analyzed for phospho-IRF3 (S396) and IRF3 immunoblots. The panels are representative data from more than three independent experiments. Veh, vehicle; Au(I), auranofin; Au(III), sodium tetrachloroaurate dihydrate.