

**Title:**

**Inhibition of Nod2 signaling and target gene expression by curcumin**

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**Abbreviations:** PAMP, pathogen-associated molecular pattern; TLRs, Toll-like receptors; PRR, pattern recognition receptors; NOD, the nucleotide-binding oligomerization domain family; LRR, leucine-rich repeat domain; NACHT, domain present in NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *podospira anserine*), and TP1 (telomerase-associated protein); iE-DAP, peptide  $\gamma$ -D-glutamyl-meso-diaminopimelic acid; MDP, muramyl dipeptide MurNAc-L-Ala-D-isoGln; NF- $\kappa$ B, nuclear factor- $\kappa$ B; I $\kappa$ B, inhibitor of NF- $\kappa$ B; RICK, Rip-like interacting CLARP kinase; CD, Crohn's disease; EGCG, epigallocatechin gallate; PTN, parthenolide.

## Abstract

Nod2 is an intracellular pattern recognition receptor that detects a conserved moiety of bacterial peptidoglycan and subsequently activates proinflammatory signaling pathways. Mutations in Nod2 have been implicated to be linked to inflammatory granulomatous disorders, such as Crohn's disease and Blau syndrome. Many phytochemicals possess anti-inflammatory properties. However, it is not known if any of these phytochemicals might modulate Nod2-mediated immune responses and thus might be of therapeutic value for the intervention of these inflammatory diseases. In this report, we demonstrate that curcumin, a polyphenol found in the plant *Curcuma longa*, and parthenolide, a sesquiterpene lactone, suppress both ligand-induced and lauric acid-induced Nod2 signaling leading to the suppression of NF- $\kappa$ B activation and target gene IL-8 expression. We provide molecular and biochemical evidence that the suppression is mediated through the inhibition of Nod2 oligomerization and subsequent inhibition of downstream signaling. These results demonstrate for the first time that curcumin and parthenolide can directly inhibit Nod2-mediated signaling pathways at the receptor level and suggest that Nod2-mediated inflammatory responses can be modulated by these phytochemicals. It remains to be determined whether these phytochemicals possess protective or therapeutic efficacy against Nod2-mediated inflammatory disorders.

## Introduction

Innate immunity provides the first line of defense against invading pathogens and is initiated by receptors that recognize pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are membrane-anchored pattern-recognition receptors (PRRs) that detect a variety of extracellular PAMPs and initiate innate immunity for host defense against pathogens (Akira and Takeda, 2004; Takeda et al., 2003). Another family of PRR called NODs (nucleotide-binding oligomerization domain-containing proteins) recognize intracellular bacterial products and activates innate immune responses in the cytosol (Fritz et al., 2006; Meylan et al., 2006). Mammals have two closely related NOD family members, Nod1 and Nod2 (Bertin et al., 1999; Inohara et al., 1999; Ogura et al., 2001b). These NOD proteins consist of three distinct domains: a C-terminal LRRs for autorepression and ligand sensing; a centrally located NACHT domain for self-oligomerization and the activation of the receptors; and an N-terminal effector domain for protein-protein interaction to initiate downstream signaling. Activation of Nod1 and Nod2 initiates proinflammatory signaling via NF- $\kappa$ B activation, necessary for clearance of infecting pathogens from the host (Carneiro et al., 2004; Inohara et al., 2005). These intracellular surveillance proteins recognize distinct motifs of bacterial peptidoglycan. Nod1 recognizes the peptide  $\gamma$ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) (Chamaillard et al., 2003; Girardin et al., 2003a) and mainly acts as a sensor for gram-negative bacteria. In contrast, Nod2 recognizes muramyl dipeptide (MDP) (Girardin et al., 2003b; Inohara et al., 2003; Tanabe et al., 2004), which is the minimal motif in all peptidoglycans, and therefore acts as a general sensor for both Gram-positive and Gram-negative bacteria.

The significance of Nod1 and Nod2 in immune responses is evident from the linkage of their mutations with inflammatory diseases in humans and the increased susceptibility of Nod1<sup>-/-</sup> and Nod2<sup>-/-</sup> mice to gastrointestinal bacterial infections. Mutations in Nod2 have been linked to susceptibility to inflammatory granulomatous disorders, such as Crohn's disease (Hugot et al., 2001; Ogura et al., 2001a) and Blau syndrome (Miceli-Richard et al., 2001). Nod2 mutations associated with Crohn's disease lead to defective NF- $\kappa$ B activation after stimulation with bacterial ligands (Girardin et al., 2003b; Inohara et al., 2003). Loss of Nod2 functions in Nod2 knockout mice abolishes the protective immunity mediated by Nod2 recognition of MDP (Kobayashi et al., 2005). In addition, Nod2 plays an important role in the activation of the adaptive immune system by acting as an adjuvant receptor for antibody production (Kobayashi et al., 2005). Nod2 mutations have also been associated with early-onset sarcoidosis, a rare but sporadic disease (Kanazawa et al., 2004; Kanazawa et al., 2005). Therefore, Nod2 plays a critical role in protecting the host from bacterial infection and loss of Nod2 functions leads to malfunctioning of the immune system and subsequently diseases.

It is now well recognized that chronic inflammation is one of the key etiological conditions for the development and progression of many chronic diseases including cancer, atherosclerosis and insulin resistance. Many phytochemicals including polyphenols and sesquiterpene lactones are known to possess anti-inflammatory properties. Curcumin, a yellow pigment present in the rhizome of turmeric (*Curcuma longa* L.) and related species, is one of the most extensively investigated phytochemicals, with regard to chemopreventive potential. Numerous studies have shown that curcumin is a potent anti-inflammatory agent (Aggarwal et al., 2007). Curcumin inhibits NF- $\kappa$ B activation induced by LPS, PMA, TNF- $\alpha$  and hydrogen peroxide through the inhibition of IKK $\beta$  that phosphorylates I $\kappa$ B $\alpha$  leading to ubiquitinylation

and degradation (Pan et al., 2000; Singh and Aggarwal, 1995). Curcumin inhibits TNF- $\alpha$ -induced cyclooxygenase-2 (COX2) expression and NF- $\kappa$ B activation in human colonic epithelial cells (Plummer et al., 1999). Curcumin also suppresses TNF- $\alpha$ -induced nuclear translocation and DNA binding of NF- $\kappa$ B in a human myeloid leukemia cell line by blocking phosphorylation and subsequent degradation of I $\kappa$ B (Aggarwal et al., 2006). More recently, curcumin has been shown to inhibit Toll-like receptor 4 (TLR4) signaling by inhibiting TLR4 homodimerization (Youn et al., 2006).

We recently demonstrated that Nod2 signaling is differentially modulated by the types of fatty acids (Zhao et al., 2007). In this report, we investigated if anti-inflammatory phytochemicals might inhibit Nod2 signaling and downstream proinflammatory gene expression. We found that curcumin and parthenolide both inhibited Nod2 signaling induced by its bacterial ligand MDP or saturated fatty acid lauric acid. The inhibition was mediated through the inhibition of Nod2 oligomerization leading to suppression of NF- $\kappa$ B activation and proinflammatory gene expression. These results demonstrated that Nod signaling pathways are amenable to be modulated by dietary phytochemicals.

## Materials and Methods

**Reagents.** Curcumin, parthenolide, helenalin, and epigallocatechin gallate (EGCG) were purchased from Biomol International (Plymouth Meeting, PA). Resveratrol and monoclonal antibodies against Flag, HA and  $\beta$ -actin were purchased from Sigma-Aldrich (St. Louis, MO). MDP was from Bachem Bioscience (King of Prussia, PA). Anti-HA affinity matrix was from Roche Applied Science (Indianapolis, IN). Polyclonal I $\kappa$ B $\alpha$  antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against NF- $\kappa$ B p65 and phospho-NF- $\kappa$ B p65 and monoclonal antibody against phospho-I $\kappa$ B $\alpha$  were purchased from Cell Signaling Technology (Danvers, MA).

**Cell Culture.** Human colonic epithelial cell line HCT116, purchased from ATCC, was cultured in McCoy's 5A medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 $\mu$ g/ml streptomycin (Invitrogen, Carlsbad, CA). HEK293T (a human embryonic kidney epithelial cell line provided by Sam Lee, Beth Israel Hospital, MA) was cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 $\mu$ g/ml streptomycin.

**Plasmids.** pcDNA3-HA-Nod2, pcDNA3-Flag-Nod2 and pcDNA3-Myc-RICK were previously described (Inohara et al., 2000; Inohara et al., 2001; Ogura et al., 2001b). (2x)-NF- $\kappa$ B luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). Human IL-8 (-173bp)-Luc was from Marie Annick Buendia (Institute Pasteur, France). pRSV- $\beta$ -galactosidase plasmid was from Jongdae Lee (University of California, San Diego, CA). Plasmid

DNA from these expression vectors was prepared in large scale for transfection using the EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA).

**Transfections and Luciferase Assays.** Transient transfections were carried out using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations. HCT116 cells were seeded  $5-10 \times 10^4$  per well of 24-well plates and co-transfected the following day with 0.2  $\mu\text{g}$  2xNF- $\kappa$ B-Luc or 0.04  $\mu\text{g}$  IL-8(-173bp)-Luc and 0.1  $\mu\text{g}$  pRSV- $\beta$ -galactosidase plasmid. For Nod2 overexpression experiments,  $5-10 \times 10^4$  HEK293T cells were seeded. 4 ng pcDNA3-Nod2-HA was co-transfected with 40 ng 2xNF- $\kappa$ B-Luc or 8 ng IL-8(-173bp)-Luc and 20 ng  $\beta$ -galactosidase control plasmid. For RICK overexpression experiments,  $5-10 \times 10^4$  HEK293T cells were co-transfected with 20 ng pcDNA3-Myc-RICK, 40 ng 2xNF- $\kappa$ B-Luc or 8 ng IL-8(-173bp)-Luc, and 20 ng  $\beta$ -galactosidase control plasmid. The total amount of plasmids was equalized by supplementation with the corresponding empty vector to minimize differences in transfection efficiency among samples. The cells were pretreated with phytochemicals for 1 hour and then coincubated with MDP or lauric acid for 6 hours before lysis. Luciferase and  $\beta$ -galactosidase enzyme activities were determined using a luciferase assay system and  $\beta$ -galactosidase enzyme assay system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by  $\beta$ -galactosidase activity to correct differences in transfection efficiency among samples. Each of the experiments was done at least three times.

**Immunoblotting and co-immunoprecipitation.** These experiments were performed essentially as previously described (Zhao et al., 2007).  $5 \times 10^6$  HCT116 cells were seeded per 100 mm dish.



The following day, the cells were pretreated with phytochemicals for 1 h and then co-incubated with MDP for the time periods indicated in the figure legends. The cells were rinsed with cold PBS twice and then lysed by sonication in cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 μg/ml leupeptin. For the NF-κB p65 translocation experiments, the nuclear and cytosolic fractions of proteins were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA) according to the supplier's protocol. The lysis supernatants were collected by centrifugation and subjected to 7.5% or 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% (v/v) Tween 20 containing 5% non-fat milk. The membrane was probed with primary antibody for 1 hr at room temperature or at 4 °C overnight followed by incubation with horse radish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. The proteins were detected by the ECL Western blot detection reagents (Amersham Biosciences, Piscataway, NJ) followed by exposure to an X-ray film (Eastman Kodak). For Nod2 oligomerization experiments, HEK293T cells were seeded 5x10<sup>6</sup> per 100 mm dish. 24 hr later the cells were transfected with 3μg pcDNA3-HA-Nod2 and 3μg pcDNA3-Flag-Nod2. 24 h after transfection the cells were rinsed twice with cold PBS and lysed in buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% triton X-100 and a Halt protease inhibitor cocktail (Pierce, Rockford, IL) by passing the cells through a 18G1 needle for 20 times. The lysate supernatants were collected by centrifugation. HA-Nod2 and Flag-Nod2 proteins in the supernatants were co-immunoprecipitated with anti-HA affinity matrix and

detected by western blotting with anti-Flag and anti-HA antibodies using the ECL Western Detection System as described above.

**Measurement of interleukin(IL)-8 protein.** The supernatants of HCT116 cells that were transiently transfected for reporter gene assays were collected and the levels of interleukin (IL)-8 protein were determined using enzyme-linked immunosorbent assay (ELISA) kits (OptEIA ELISA kits, BD Pharmingen, San Diego, CA) according to the supplier's instructions.

**Data Analysis.** Data from the reporter and ELISA assays were analyzed by a 2-tailed Student T-test.

## Results

### **Curcumin inhibited Nod2 ligand-induced NF- $\kappa$ B activation and target gene IL-8**

**expression.** Nod2 signaling leads to the activation of the transcription factor NF- $\kappa$ B, which in turn induces the expression of proinflammatory genes (Strober et al., 2006). Many phytochemicals possess anti-inflammatory properties. We were interested in knowing if any of these phytochemicals might inhibit Nod2 signaling thereby suppressing the inflammatory responses. We chose a panel of phytochemicals consisting of curcumin, parthenolide, helenalin, epigallocatechin gallate (EGCG), and resveratrol. Curcumin has been well documented to exhibit potent anti-inflammatory, anti-viral, and anti-bacterial activities (Aggarwal et al., 2007). To test whether curcumin downregulated Nod2 signaling, we looked at the effects of curcumin on Nod2 ligand-induced NF- $\kappa$ B activation and target gene IL-8 expression using NF- $\kappa$ B and IL-8 luciferase reporter genes as readouts. As shown in Figure 1, curcumin inhibited MDP-induced NF- $\kappa$ B activation in a dose-dependent manner. Similar inhibitory effects on MDP-induced NF- $\kappa$ B activation were also observed for two other phytochemicals, parthenolide and helenalin. In contrast, EGCG (a polyphenol rich in green tea) and resveratrol (a polyphenol rich in grape) exhibited no inhibitory effects on MDP-induced NF- $\kappa$ B activation. In fact, resveratrol enhanced MDP-induced NF- $\kappa$ B activation. Consistent with NF- $\kappa$ B reporter assays, curcumin and parthenolide inhibited the transactivation of IL-8 expression. This inhibition was detected by both the reporter assays for transcription activation and ELISA assays for the IL-8 protein secreted from the cells (Figure 2 A, B, D, E). In contrast, EGCG showed little inhibition on IL-8 reporter transactivation and IL-8 protein expression (Figure 2 C, F). These results demonstrate that curcumin and parthenolide negatively modulate MDP-induced NOD2 signaling leading to

the downregulation of NF- $\kappa$ B activation and the suppression of proinflammatory gene expression.

**Curcumin inhibited lauric acid (C12:0)-induced Nod2 activation.** The innate immune systems were evolved to defend host against pathogenic infections. It has now been well documented that the activation of certain pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and NOD proteins (Nods), are also modulated by endogenous molecules, including fatty acids (Lee and Hwang, 2006; Zhao et al., 2007). For example, saturated fatty acids induce the activation of Nods signaling whereas unsaturated fatty acids, particularly n-3 polyunsaturated fatty acids, inhibit the activation of Nods signaling induced by saturated fatty acids or Nod ligands (Zhao et al., 2007). To determine if curcumin also inhibits the activation of Nod2 signaling induced by saturated fatty acids we coincubated HCT116 cells with saturated fatty acid lauric acid (C12:0) and curcumin. As shown in Figure 3, curcumin dose dependently inhibited C12:0-induced NF- $\kappa$ B activation (Figure 3A). Curcumin also inhibited C12:0-induced transactivation of IL-8-Luc reporter and the expression of IL-8 protein (Figure 3B-C). Similar inhibition was observed for parthenolide on C12:0-induced NF- $\kappa$ B activation and IL-8 reporter transactivation and protein expression. Thus, curcumin and parthenolide inhibited not only the activation of Nod2 signaling induced by MDP, a Nod2 ligand of bacterial origin, but also the activation of Nod2 signaling induced by saturated fatty acid lauric acid (C12:0).

**Curcumin inhibited MDP-induced phosphorylation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65.** The activation of Nod2 signaling leading to the expression of proinflammatory genes is mediated by the phosphorylation of a cascade of effector proteins, including I $\kappa$ B $\alpha$  and NF $\kappa$ B p65 (Strober et al.,

2006). Phosphorylation of these effector proteins leads to the release of the active form of NF- $\kappa$ B, which in turn translocates to the nucleus and activates the expression of its target pro-inflammatory genes. MDP induced I $\kappa$ B $\alpha$  phosphorylation with a peak at two hours after MDP treatment. Treatment of the cells with curcumin dramatically inhibited MDP-induced phosphorylation of this effector protein (Figure 4A). During the two-hour incubation, curcumin dose-dependently inhibited MDP-induced I $\kappa$ B $\alpha$  phosphorylation (Figure 4B). In contrast, EGCG showed no significant effect on MDP-induced I $\kappa$ B $\alpha$  phosphorylation (Figure 4B). The inhibitory effect by curcumin on phosphorylation of I $\kappa$ B $\alpha$  was confirmed by its inhibition on I $\kappa$ B $\alpha$  degradation (data not shown). Curcumin treatment also significantly inhibited MDP-induced phosphorylation of the effector protein NF- $\kappa$ B p65 in a similar dose-dependent manner, whereas, EGCG had no inhibitory effect (Figure 4C-D). Similarly, treatment of the cells with parthenolide also inhibited MDP-induced phosphorylation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 in a dose-dependent manner (Figure 4E-F). Consistent with the inhibition of NF- $\kappa$ B p65 phosphorylation, curcumin dose-dependently decreased the levels of NF- $\kappa$ B p65 translocated to the nucleus (Figure 4G). Similar inhibitory effects on p65 translocation were observed for parthenolide (data not shown).

**Curcumin inhibited Nod2 oligomerization.** The inhibition of MDP-induced phosphorylation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 by curcumin and parthenolide suggested that these phytochemicals might directly inhibit the phosphorylation of these effector proteins. Alternatively, they might inhibit a target protein upstream of these effector proteins, the suppression of which in turn led to the inhibitory effects on the phosphorylation of these effector proteins. To identify the target for the inhibition of Nod2 signaling by curcumin and parthenolide, we used a reconstituted system in

which 293T cells had been transiently transfected with Nod2. 293T cells express only trace amount of Nod2 (Zhao et al., 2007). As shown in Figure 5, overexpression of Nod2 in 293T cells stimulated Nod2 signaling leading to increased NF- $\kappa$ B activation. Treatment of the cells with curcumin inhibited Nod2 overexpression-induced NF- $\kappa$ B activation in a dose-dependent manner (Figure 5A). MDP treatment of 293T cells overexpressing Nod2 further enhanced NF- $\kappa$ B activation, and curcumin dose-dependently inhibited this MDP-induced NF- $\kappa$ B activation (Figure 5B). Similar to curcumin, parthenolide also inhibited MDP-induced NF- $\kappa$ B reporter expression in a dose dependent manner in Nod2 overexpressing 293T cells (Figure 5C). These data indicated that curcumin and parthenolide also inhibited NF- $\kappa$ B activation induced by Nod2 overexpression or MDP stimulation in 293T cells overexpressing Nod2.

Next, we examined if curcumin and parthenolide inhibited RICK-induced Nod2 signaling in 293T cells that overexpress RICK. As shown in Figure 5D-E, overexpression of RICK in 293T cells stimulated Nod2 signaling resulting in enhanced NF- $\kappa$ B activation. However, treatment of the cells with curcumin or parthenolide had no significant effects on the induction of NF- $\kappa$ B reporter expression by RICK overexpression. These data suggest that the target for the inhibition of Nod2 signaling by curcumin and parthenolide might be located at the upstream of Nod2-RICK interaction instead of downstream of RICK.

To test if curcumin inhibited Nod2 oligomerization, we cotransfected 293T cells with HA-tagged Nod2 and Flag-tagged Nod2 cDNA expression vectors. The resulting Nod2 proteins were coimmunoprecipitated with anti-HA antibody matrix and detected by immunoblotting with anti-Flag and anti-HA antibodies (Figure 6). MDP induced Nod2 oligomerization with a peak

induction at 100 ng/ml MDP and 15 min after MDP treatment (Zhao et al., 2007). These conditions were used for the coimmunoprecipitation experiments. As shown in Figure 6A, curcumin inhibited MDP-induced Nod2 oligomerization in a dose dependent manner. Similarly, curcumin also inhibited Nod2 oligomerization induced by lauric acid in a dose dependent manner (Figure 6B). Similar inhibition of Nod2 oligomerization was observed for parthenolide (data not shown). The fact that curcumin inhibited Nod2 oligomerization, the first step of Nod2 signaling, but not RICK overexpression-induced NF- $\kappa$ B activation, the downstream event of Nod2 signaling, strongly suggests that the target for the inhibition of Nod2 signaling by curcumin and parthenolide is Nod2 oligomerization.

## Discussion

We show here that dietary phytochemical compounds curcumin (a polyphenol) and parthenolide (a sesquiterpene lactone) inhibited NF- $\kappa$ B activation and target gene IL-8 expression. The inhibitory effects were mediated through the inhibition of Nod2 oligomerization and subsequent inhibition of downstream signaling.

Numerous studies have demonstrated the inhibitory effects of curcumin on NF- $\kappa$ B pathway as one of the key mechanisms underlying its anti-inflammatory and anti-cancer properties (Jobin et al., 1999; Kang et al., 2004; Pan et al., 2000; Plummer et al., 1999; Shishodia et al., 2005; Shishodia et al., 2003; Singh and Aggarwal, 1995). For example, it was reported that curcumin inhibited cytokine IL-1 $\beta$ -induced NF- $\kappa$ B activation and proinflammatory gene expression by inhibiting IKK activity (Jobin et al., 1999). However, the inhibition of IKK activity by curcumin was not mediated by direct interference with IKK activity, implying that the inhibition occurs rather at the upstream signal event(s) leading to reduced IKK activity. Moreover, curcumin inhibited both ligand-induced and ligand-independent dimerization of TLR4, an prerequisite step in TLR4 activation, leading to inhibition of LPS (TLR4 agonist)-induced NF- $\kappa$ B activation (Youn et al., 2006). Here, we show that curcumin also inhibits ligand dependent-Nod2 oligomerization, leading to reduced downstream NF- $\kappa$ B activation and target gene expression.

As a member of NOD-LRR family, Nod2 has recently been implicated to be involved in cytosolic innate recognition of bacteria and host defense (Inohara et al., 2005). Moreover,



mutations of the Nod2 gene have been associated with susceptibility to inflammatory diseases. Because of the deficiency in sensing bacterial PGN or synthetic MDP, the three major Nod2 mutations, R702W, G908R and L1007fsinsC, that have been associated with Crohn's disease, a chronic relapsing inflammatory disease of the bowel, represent loss-of-function mutations (Hugot et al., 2001; Ogura et al., 2001a). In contrast, the Nod2 mutations R334Q, R334W and L469F, that have been associated with the development of Blau syndrome, a dominantly inherited disease characterized by early-onset granulomatous arthritis, uveitis, and skin rashes, exhibit constitutive NF- $\kappa$ B activity compared to the wild-type Nod2. Thus, these mutations represent gain-of-function mutations (Miceli-Richard et al., 2001). Furthermore, Nod2 has been shown to mediate "sterile" inflammation induced by non-microbial molecules (Zhao et al., 2007). Therefore, it is of worth to determine whether curcumin and parthenolide are of therapeutic value in treating these Nod2-mediated inflammatory conditions, particularly Blau syndrome, which seems to be induced by constitutive activation of Nod2 in the absence of pathogenic infection.

The mechanisms by which curcumin and parthenolide inhibit Nod2 receptor oligomerization remain to be determined. Many polyphenol compounds including curcumin containing  $\alpha,\beta$ -unsaturated carbonyl group can react with biological nucleophiles such as sulfhydryl group by Michael addition (Dinkova-Kostova et al., 2001; Fang et al., 2005; Rungeler et al., 1999; Siedle et al., 2004). For example, curcumin has been shown to bind to catalytically active cysteine residue of thioredoxin reductase by Michael addition to inhibit the enzyme activity (Fang et al., 2005). Nod2 contains a centrally located nucleotide-binding oligomerization (NOD) domain that mediates self-oligomerization, in addition to a C-terminal

LRR domain for ligand recognition and an N-terminal CARD domain for effector binding. Sequence analysis reveals several cysteine residues (Cys<sup>333</sup>, Cys<sup>353,354</sup> and Cys<sup>395</sup>) in the NOD domain, which might mediate Nod2 oligomerization by forming disulfide bonds. Whether curcumin inhibits Nod2 oligomerization by disrupting the cysteine-mediated disulfide bonds between the NOD domains remains to be determined. The results that curcumin inhibits both TLR4- and Nod2-mediated signaling pathways by inhibiting their oligomerization provide new insights into the mechanisms that underlie the anti-inflammatory effects of curcumin.

In conclusion, we show that curcumin and parthenolide inhibit Nod2-mediated NF- $\kappa$ B activation and target gene expression by inhibiting Nod2 oligomerization. Our results suggest a possibility that curcumin and parthenolide might be beneficial in treating Nod2-mediated inflammatory conditions, including Blau syndrome.

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## Footnotes

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## Figure legends

Fig 1. Curcumin inhibits MDP-induced NF- $\kappa$ B activation. HCT116 cells were transfected with NF- $\kappa$ B-luciferase and  $\beta$ -galactosidase reporters. After 24 hr, the cells were pretreated with curcumin (10, 20, 30  $\mu$ M), parthenolide (5, 10, 15  $\mu$ M), helenalin (1, 3, 5  $\mu$ M), EGCG (10, 30, 50  $\mu$ M) or resveratrol (10, 30, 50  $\mu$ M) for 1 h and then co-incubated with 50  $\mu$ M MDP for additional 6 h. Cell lysates were prepared and luciferase and  $\beta$ -galactosidase enzyme activities measured as described in Materials and Methods section. Relative luciferase activity (RLA) was normalized with  $\beta$ -galactosidase activity. Values are mean  $\pm$  S.E.M (n=3). Statistical significant difference (p<0.05) is indicated between cells treated with MDP alone and cells treated with MDP plus the indicated phytochemicals. MDP, muramyl dipeptide; Cur, curcumin; PTN, parthenolide; Hel, helenalin; EGCG, epigallocatechin gallate; Res, resveratrol. The molecular structure is shown above the reporter assay data for each of the phytochemicals.

Fig 2. Curcumin inhibits MDP-induced transactivation of IL-8. HCT116 cells were co-transfected with IL-8-luciferase and  $\beta$ -galactosidase reporter constructs. After 24 hr, the cells were pretreated with curcumin (10, 20, 30  $\mu$ M) (A, D), parthenolide (5, 10, 15  $\mu$ M) (B, E), or EGCG (10, 30, 50  $\mu$ M) (C, F) for 1 h and then co-incubated with 50  $\mu$ M MDP for additional 6 h. Cell lysates (A-C) were prepared and luciferase and  $\beta$ -galactosidase enzyme activities were measured as described in Materials and Methods section. Medium supernatants (D-F) were collected from IL-8-luciferase transfected cells and the levels of IL-8 protein were measured as described in Materials and Methods section. Relative luciferase activity (RLA) was normalized with  $\beta$ -galactosidase activity. Values are mean  $\pm$  S.E.M (n=3). Statistical significant difference

( $p < 0.05$ ) is indicated between cells treated with MDP alone and cells treated with MDP plus the indicated phytochemicals. Abbreviations are the same as described in Figure 1.

Fig 3. Curcumin inhibits C12:0-induced transactivation of NF- $\kappa$ B and IL-8 expression.

HCT116 cells were co-transfected with NF- $\kappa$ B-luciferase or IL-8-luciferase and  $\beta$ -galactosidase reporter constructs. After 24 hr, the cells were pretreated with curcumin (10, 20, 30  $\mu$ M) (A-C) or parthenolide (5, 10, 15  $\mu$ M) (D-F) for 1 h and then co-incubated with C12:0 (100  $\mu$ M) for additional 6 h. Cell lysates were prepared and luciferase and  $\beta$ -galactosidase enzyme activities were measured as described in Materials and Methods section. Medium supernatants were collected from IL-8-luciferase transfected cells and the levels of IL-8 protein were measured as described in Materials and Methods section. Relative luciferase activity (RLA) was normalized with  $\beta$ -galactosidase activity. Values are mean  $\pm$  S.E.M (n=3). Statistical significant difference ( $p < 0.05$ ) is indicated between cells treated with MDP alone and cells treated with MDP plus the indicated phytochemicals. Abbreviations are the same as described in Figure 1.

Fig 4. Curcumin inhibits MDP-induced phosphorylation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65. (A-F) HCT cells were pretreated with 30  $\mu$ M curcumin (Cur) (A, C) or 15  $\mu$ M parthenolide (PTN) (E) for 1 hr and then coincubated with 50  $\mu$ M MDP for 0-240 min or pretreated with curcumin (10, 20, 30  $\mu$ M) (B, D), EGCG (10, 30, 50  $\mu$ M) (B, D) or parthenolide (5, 10, 15  $\mu$ M) (F) for 1 hr then coincubated with 50  $\mu$ M MDP for 2 hr. The protein lysates were prepared and analyzed by western blotting using anti-phospho-I $\kappa$ B $\alpha$  (p-I $\kappa$ B $\alpha$ ), anti-phospho NF- $\kappa$ B p65 (p-p65), and anti-NF- $\kappa$ B p65 (p65) antibodies as described in Materials and Methods section. (G) HCT cells were pretreated with curcumin (10, 20, 30  $\mu$ M) for 1 hr then coincubated with 50  $\mu$ M MDP for 2 hr.



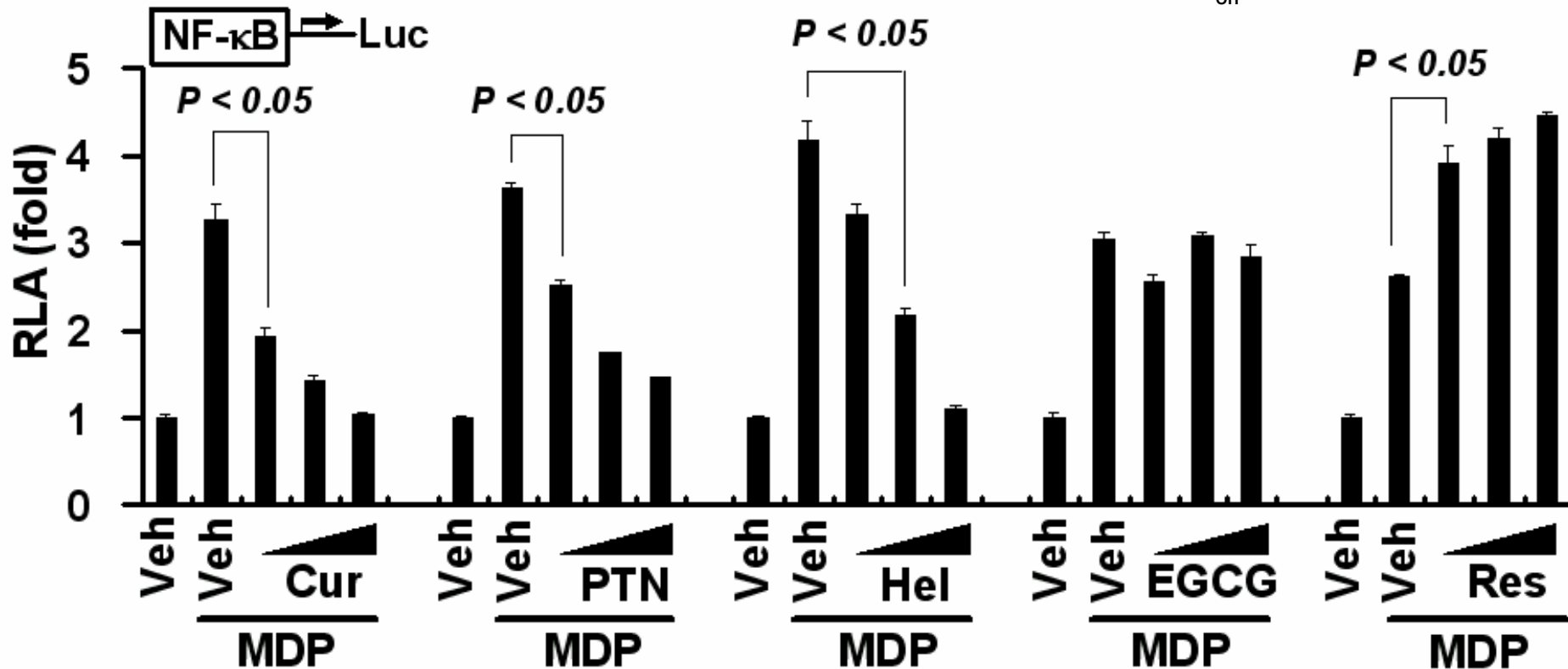
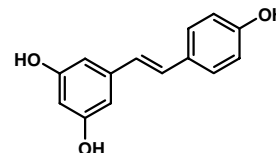
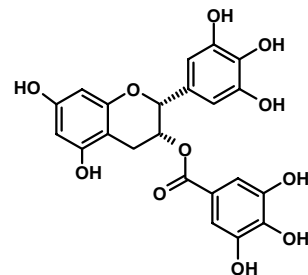
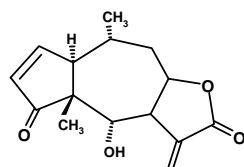
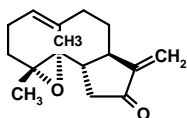
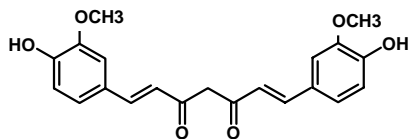
Nuclear (nucl) and cytosolic (cyto) fractions of protein lysates were prepared and analyzed by western blotting using anti-phospho NF- $\kappa$ B p65 (p-p65), and anti- NF- $\kappa$ B p65 (p65) antibodies as described in Materials and Methods section.  $\beta$ -actin was used as loading controls.

Fig 5. Curcumin does not inhibit RICK-induced NF- $\kappa$ B activation. (A-C) HEK293T cells were co-transfected with NF- $\kappa$ B-luciferase and  $\beta$ -galactosidase reporter constructs and Nod2 expression vector. 24 h after transfection, the cells were treated with curcumin (10, 20, 30  $\mu$ M) for 6 h (A), or pretreated with curcumin (10, 20  $\mu$ M) for 1 h and then coincubated with 200 ng/ml MDP for additional 6 h (B), or pretreated with parthenolide (5, 10, 15  $\mu$ M) for 1 h and then coincubated with 200 ng/ml MDP for additional 6 h (C). (D-E) HEK293T cells were co-transfected with RICK expression vector and NF- $\kappa$ B-luciferase and  $\beta$ -galactosidase reporters. 24 h after transfection, the cells were treated with curcumin (10, 20, 30  $\mu$ M) (D) or parthenolide (5, 10, 15  $\mu$ M) (E) for 6 h. Cell lysates were prepared and luciferase and  $\beta$ -galactosidase enzyme activities were measured as described in Materials and Methods section. Relative luciferase activity (RLA) was normalized with  $\beta$ -galactosidase activity. Values are mean  $\pm$ S.E.M (n=3). Statistical significant difference (p<0.05) is indicated between cells treated with vehicle and cells treated with curcumin (A), or between cells treated with MDP alone and cells treated with MDP plus curcumin (B) or parthenolide (C). Abbreviations are the same as described in Figure 1.

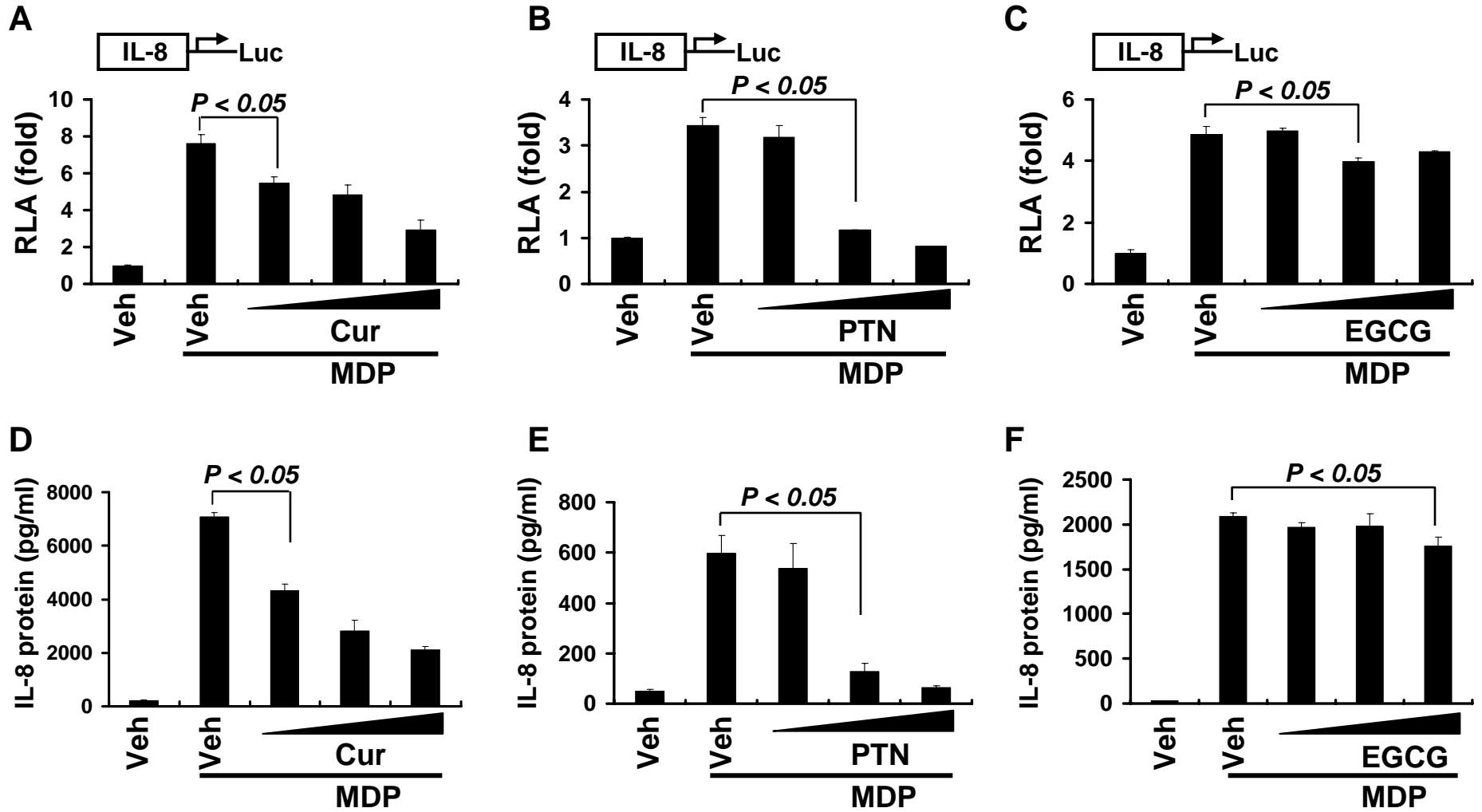
Fig 6. Curcumin inhibits Nod2 oligomerization induced by MDP or C12:0. HEK293T cells were cotransfected with HA-Nod2 and Flag-Nod2 cDNA expression vectors. After 24 h cells were pretreated with curcumin (10, 20, 30  $\mu$ M) for 1 h and then co-incubated with 100 ng/ml MDP (A) or 100  $\mu$ M C12:0 (B) for 15 min. Cell lysates were prepared, Nod2 proteins were

immunoprecipitated with anti-HA affinity matrix, and HA-Nod2 and Flag-Nod2 proteins were detected by western blotting using anti-HA and anti-Flag antibodies as described in Materials and Methods section. IP, immunoprecipitation; IB, immunoblotting.

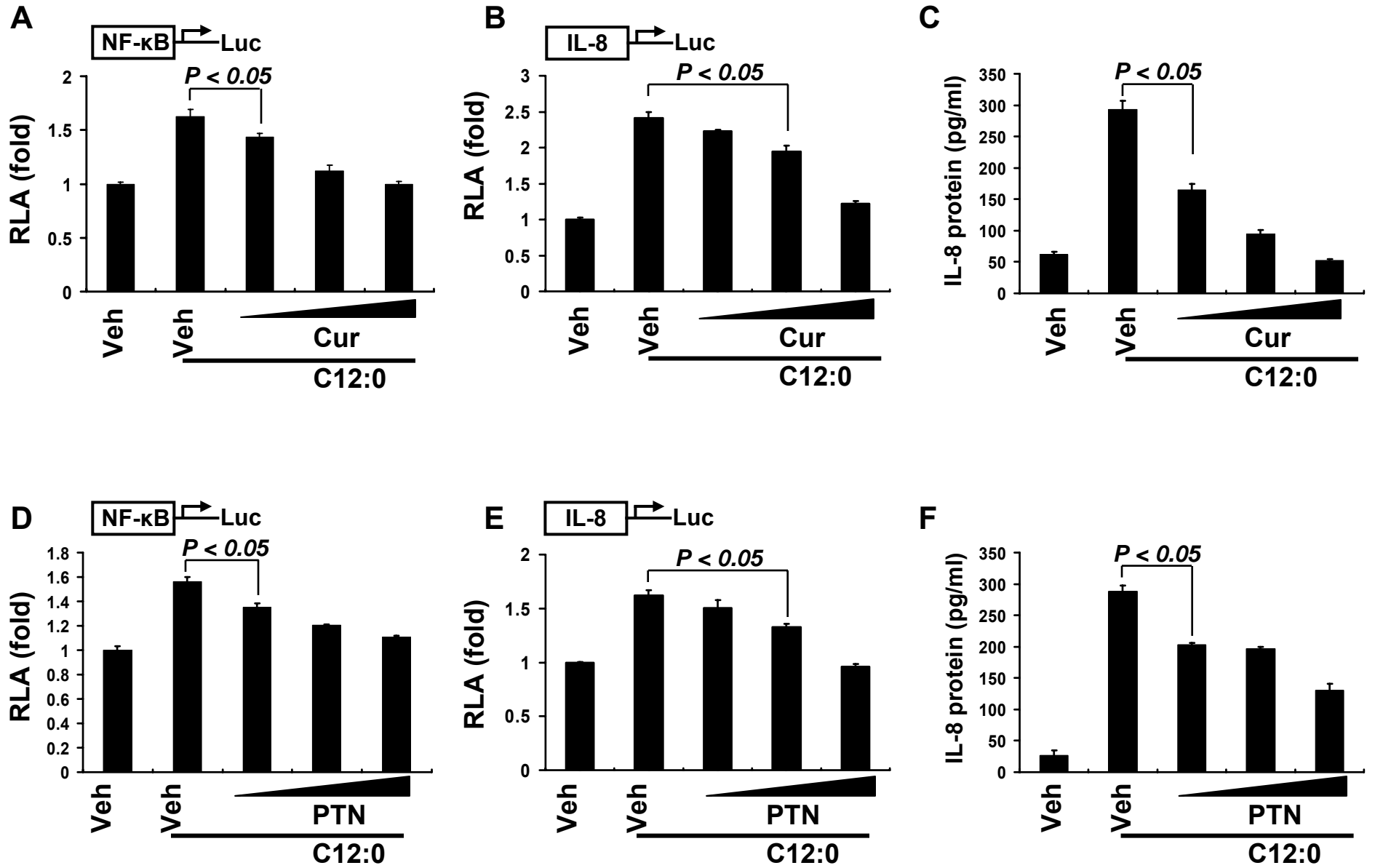
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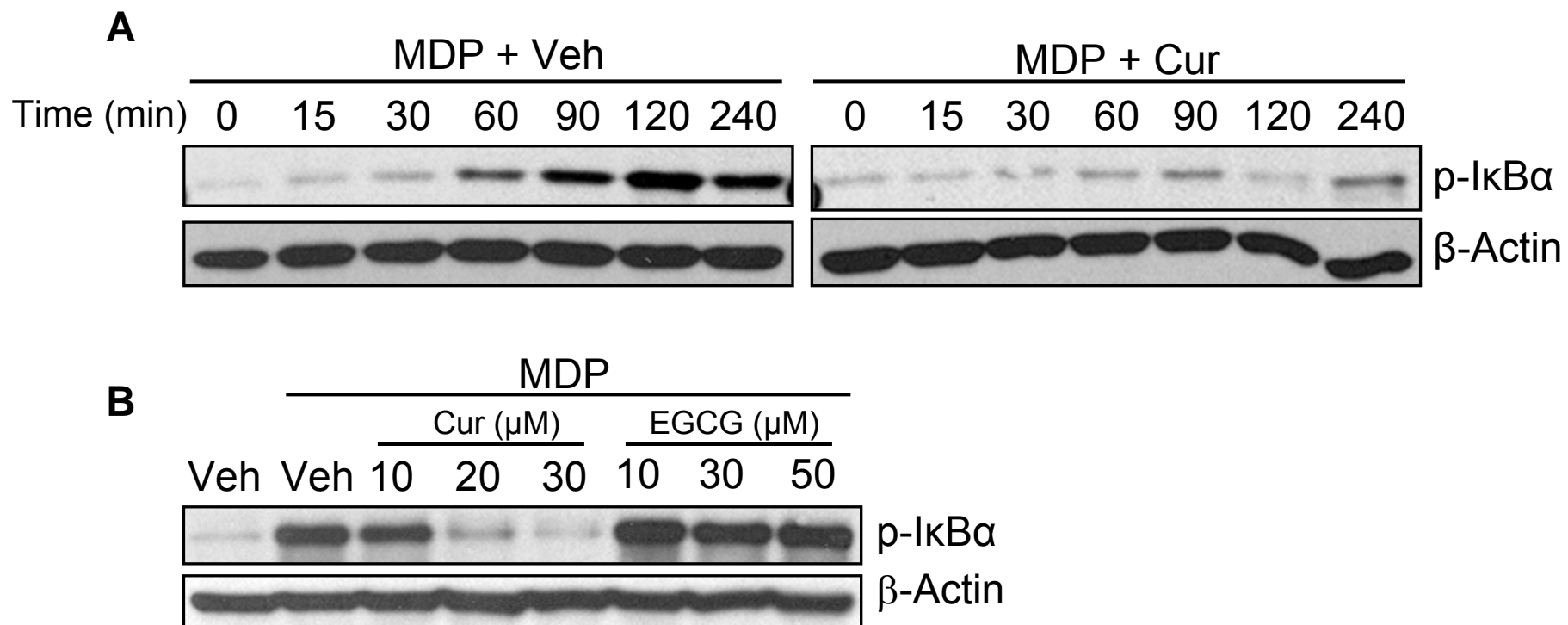
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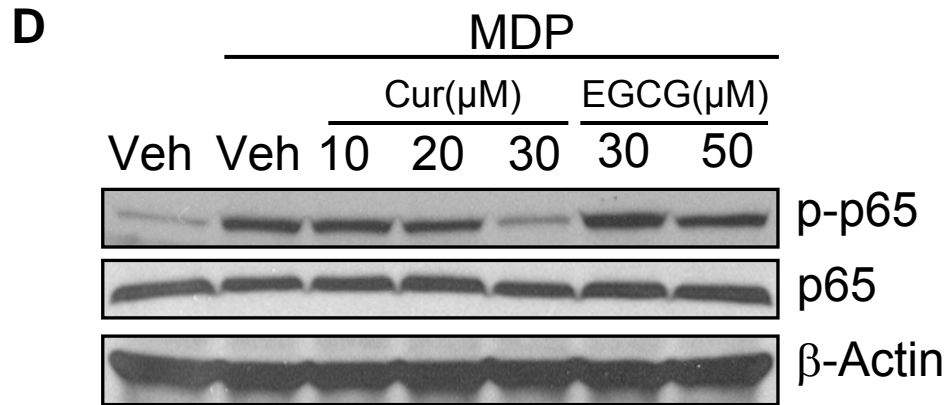
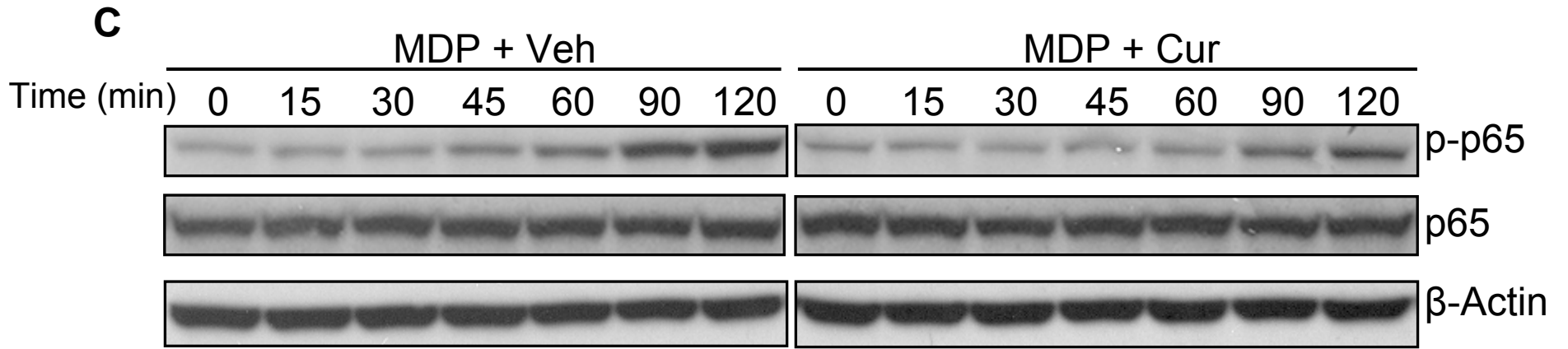
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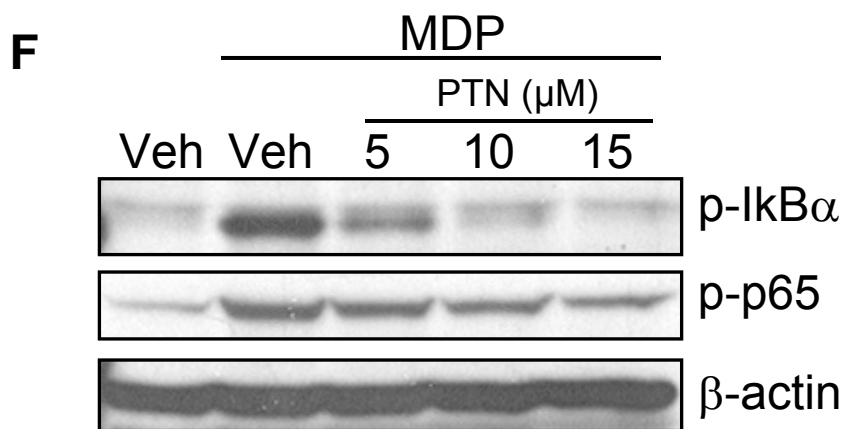
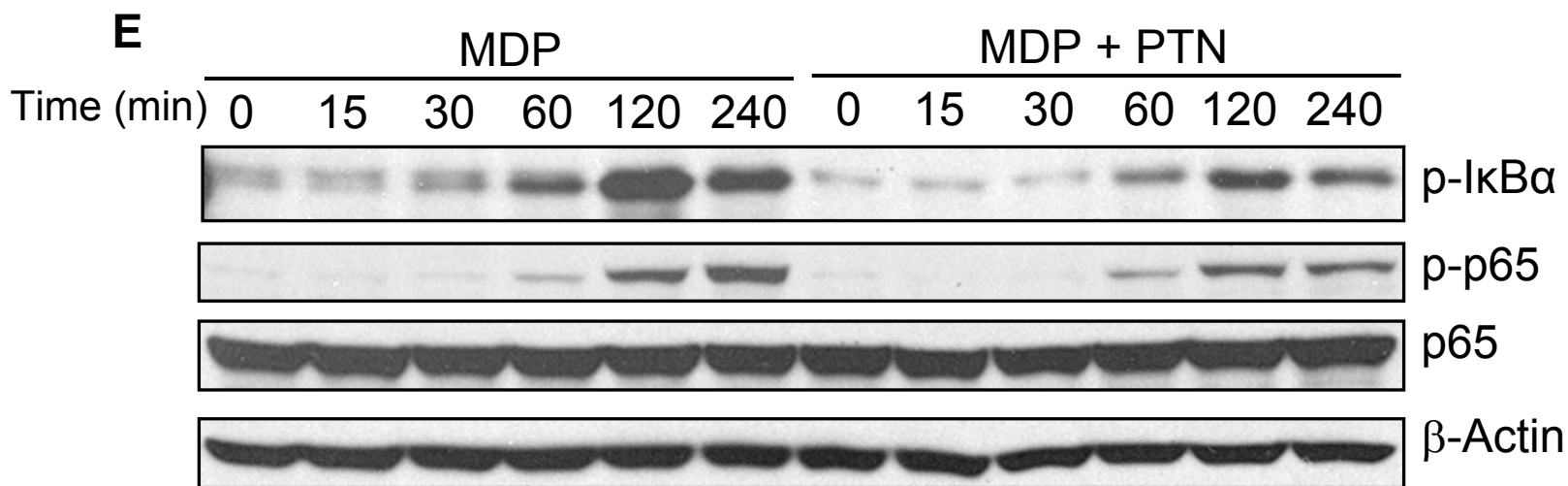
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MOL #46169 Figure 4 C-D

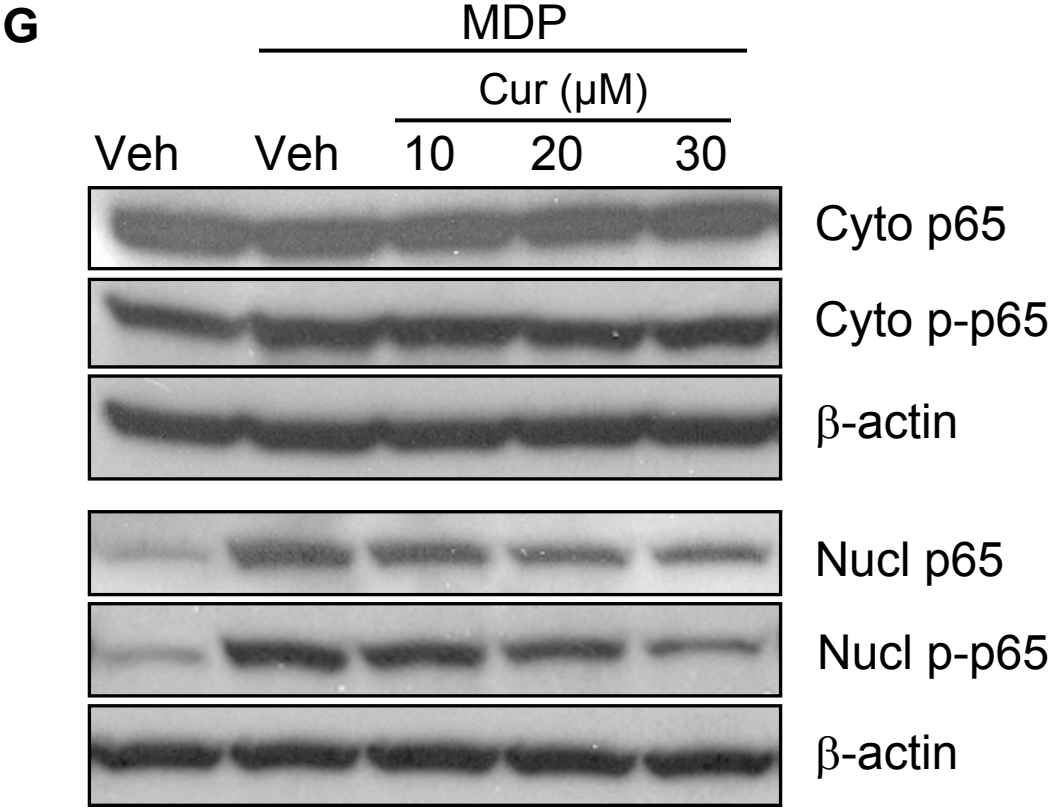


MOL #46169 Figure 4 E-F

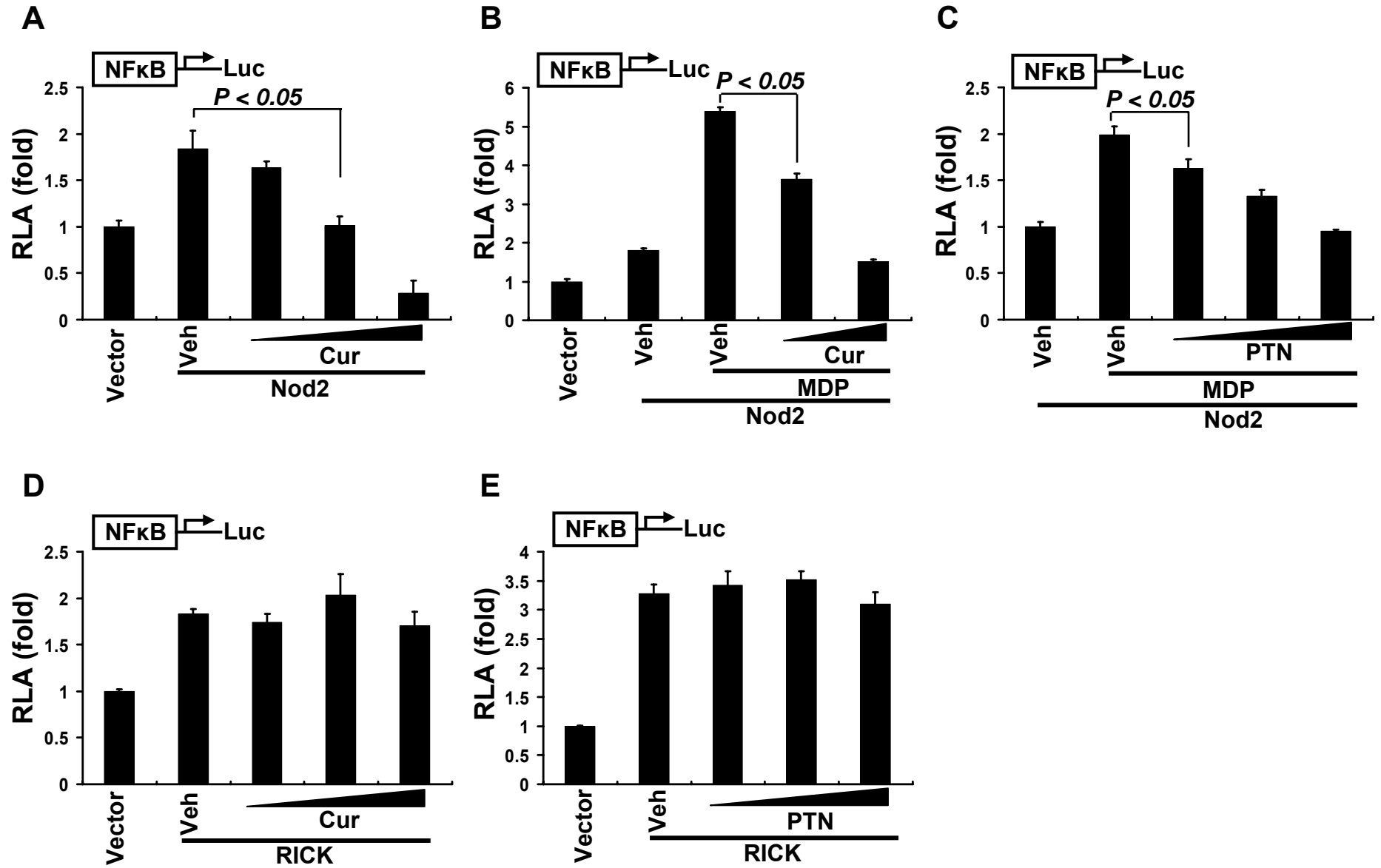




MOL #46169 Figure 4 G



MOL #46169 Figure 5



MOL #46169 Figure 6

