

Treatment of THP-1 cells with *Uncaria tomentosa* extracts differentially regulates the expression of IL-1 β and TNF- α

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Abstract

Uncaria tomentosa, commonly known as cat's claw, is a medicinal plant native to Peru, which has been used for decades in the treatment of various inflammatory disorders. *Uncaria tomentosa* can be used as an antioxidant, has anti-apoptotic properties, and can enhance DNA repair, however it is best known for its anti-inflammatory properties. Treatment with *Uncaria tomentosa* extracts inhibits the production of the pro-inflammatory cytokine, TNF- α , which is a critical mediator of the immune response. In this paper, we showed that treatment of THP-1 monocyte-like cells with *Uncaria tomentosa* extracts inhibited the MAP kinase signaling pathway and altered cytokine expression. Using ELISA assays, we showed that treatment with *Uncaria tomentosa* extracts augmented LPS-dependent expression of IL-1 β by 2.4-fold, while inhibiting the LPS-dependent expression of TNF- α by 5.5-fold. We also showed that treatment of LPS-stimulated THP-1 cells with *Uncaria tomentosa* extracts blocked ERK1/2 and MEK1/2 phosphorylation in a dose-dependent manner. These data demonstrate that treatment of THP-1 cells with *Uncaria tomentosa* extracts has opposite effects on IL-1 β and TNF- α secretion, and that these changes may involve effects on the MAP kinase pathway.
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1. Introduction

Uncaria tomentosa (Willd.) DC (Rubiaceae), commonly known as Uña de gato or cats claw, is a medicinal plant that was traditionally used by the Ashaninka First Nation of Amazonian Peru. Because of the high regard with which it is held by traditional healers, Peruvians have used it in a bark infusion for decades to treat disorders such as inflammatory diseases, arthritis, cancer, gastric ulcers, recovery from childbirth, and control of inflammation (Akesson et al., 2003a; Heitzman et al., 2005). The medicinal extract has been commercialized and is currently in widespread use across North America.

Uncaria tomentosa extracts can act as an antioxidant and are able to enhance DNA repair (Sandoval et al., 2000; Miller et al., 2001; Akesson et al., 2003a; Goncalves et al., 2005; Mammone et al., 2006; Pilarski et al., 2006). *In vitro* studies have shown that *Uncaria tomentosa* may have an anti-apoptotic activity in some cells, such as lymphocytes, following treatment with apoptotic inducers such as hydrogen peroxide, diphenyl-2-picrylhydrazyl, and peroxyntirite (Sheng et al., 1998; Miller et al., 2001; Akesson et al., 2003a,b). This increase in cell viability may be due to an enhancement of DNA repair in damaged cells (Lamm et al., 2001; Sheng et al., 2001; Mammone et al., 2006) and could be very significant for treatment of patients undergoing chemotherapy. In fact, patients treated with combination chemotherapy including *Uncaria tomentosa* showed accelerated recovery in white blood cell counts as well as decreased side effects such as hair loss, weight loss, nausea, and secondary infections (Steinberg, 1995). However, the effects of *Uncaria*

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tomentosa depend on the cell type studied. For example, Raji cells treated with *Uncaria tomentosa* are protected from apoptosis, whereas *Uncaria tomentosa* induces apoptosis in some human leukemia cell lines (Akesson et al., 2003a).

Treatment with *Uncaria tomentosa* extracts also inhibits the production of tumor necrosis factor- α (TNF- α) (Sandoval et al., 2000; Setty and Sigal, 2005). TNF- α is a potent pro-inflammatory cytokine and is a critical mediator of chronic inflammatory conditions including rheumatoid arthritis (Piscoya et al., 2001). TNF- α is cytotoxic to lymphocytes and in some situations can act to suppress the immune system. The release of TNF- α from monocytes is stimulated by treatment with various chemical agents, including bacterial lipopolysaccharide (LPS) (Aguilar et al., 2002). LPS is a potent bacterial endotoxin that is shed from the outer membrane of gram-negative bacteria and that acts as an immune system stimulant. In cells pre-treated with *Uncaria tomentosa* and stimulated by treatment with LPS, TNF- α production was suppressed by 65–85% compared to cells treated only with LPS (Sandoval et al., 2000; Sheng et al., 2000a; Piscoya et al., 2001; Aguilar et al., 2002). The ability of *Uncaria tomentosa* extracts to suppress TNF- α release occurs at concentrations four orders of magnitude lower than its anti-oxidant potential (Sandoval et al., 2000). This suggests that the medicinal effect of *Uncaria tomentosa* may be mediated via alteration in immune responsiveness.

It is thought that the inhibition of TNF- α expression following treatment with *Uncaria tomentosa* extracts is controlled by the regulation of the transcription factor NF κ B (Sandoval et al., 2000; Akesson et al., 2003b; Mammone et al., 2006; Pilarski et al., 2006). NF κ B can be activated by several different signals, and can regulate the expression of several pro-inflammatory cytokines including TNF- α , IL-1, IL-2, IL-6, and IL-8 (Akesson et al., 2003b). NF κ B activation also promotes the expression of anti-apoptosis genes and the proliferation of lymphocytes. However, treatment with *Uncaria tomentosa* has contradictory activities since it can inhibit the activation of NF κ B and can also activate anti-apoptosis mechanisms that protect some cell types, such as lymphocytes, from apoptosis (Akesson et al., 2003a,b). In this report, we showed that treatment with *Uncaria tomentosa* extracts inhibited LPS-dependent TNF- α secretion but enhanced LPS-dependent IL-1 β secretion. Further, treatment with *Uncaria tomentosa* extracts strongly inhibited activation of the MAP kinase pathway but did not enhance cell death.

2. Materials and methods

2.1. Plant material

Bark was collected from wild *Uncaria tomentosa* plants grown in the lowland Peruvian Amazon Region, Iquitos. A voucher has been placed in the herbarium at the University of Ottawa. The bark was dried and ground, producing 253.7 g of plant material. The component compounds were extracted by exhaustive percolation with 95% ethanol. The solvent was then evaporated at reduced pressure with a rotavapour at a temperature less than 40 °C. The yield was 41.7 g of ethanol-extracted

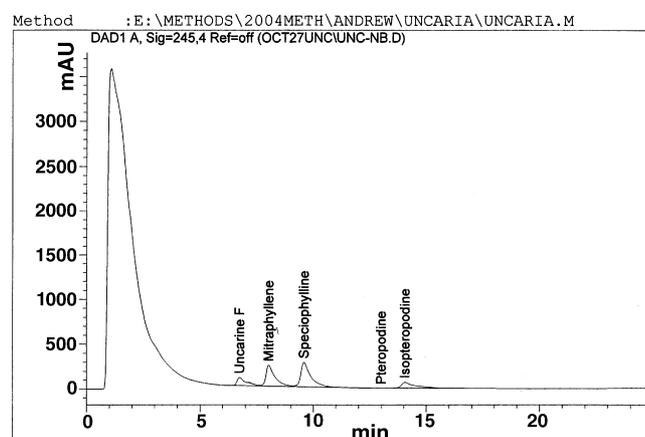


Fig. 1. HPLC analysis of *Uncaria tomentosa*.

product. The stock solution was then diluted in RPMI media prior to use in tissue culture.

2.2. HPLC analysis

Uncaria tomentosa extracts were filtered with a 0.2 μ m filter prior to HPLC analysis. Separation was achieved using a 125 mm \times 4.6 mm \times 18.5 μ m Superspher column with a 4 mm guard. The oven temperature was set at 45 °C with a flow rate of 1 ml/min. The solvents used were; A: 5 nM Na₂HPO₄, 5 nM KH₂PO₄, pH 6.6, and B: 1:1 MeOH:MeCN. The solvent program was 40–70% B in 30 min, 70–80% B in 2 min, hold at 80% B for 10 min, 80–40% B in 3 min and equilibrate 15 min. The detection wavelength was 245 nm (Fig. 1). Standards were obtained from our collection and from Cerilliant (Round Rock, TX) (Table 1).

2.3. Experimental protocol

THP-1 cells (ATCC, Rockville, MD) were grown at 37 °C in 5% CO₂ in RPMI media supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 100 μ g/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Burlington, ON) to 1 \times 10⁶ cells/ml. Cells were collected by centrifugation and resuspended at a concentration of 2 \times 10⁶ cells/ml in serum-free RPMI media. Cells were treated in the presence or absence of *Uncaria tomentosa* extracts and incubated at 37 °C in 5% CO₂ for 24 h. Samples were then treated in the presence or absence of *Uncaria tomentosa* extracts and/or 5 μ g/ml of *Escherichia coli* LPS (serotype 0127, Sigma Chemical, St. Louis, MO), and incubated for 24 h.

Table 1

Compounds found by HPLC in *Uncaria tomentosa* extract used experimentally

| Compound name | Retention time (min) | Peak area | Amount (μ g/ml) |
|------------------|----------------------|-----------|----------------------|
| Uncarine F | 6.75 | 1927 | 145.4 |
| Mitraphyllene | 8.04 | 6621 | 387.9 |
| Speciophylline | 9.59 | 8617 | 469.6 |
| Isomitraphyllene | 0 | 0 | 0 |
| Pteropodine | 12.99 | 3 | 0.3 |
| Isopteropodine | 14.06 | 2571 | 185.1 |

2.4. Flow cytometer analysis

Samples, stained in 0.1% propidium iodide in methanol as described (Guo and Lee, 1999), were analyzed on a Beckman Coulter Epics Elite flow cytometer.

2.5. Trypan blue analysis

Samples were stained with 0.1% of the vital dye trypan blue, and then examined on a hemocytometer. Dead cells were identified by lack of exclusion of stain.

2.6. Protein isolation and immunoblot analysis

Cells were collected by centrifugation and lysed in RIPA buffer (1% Triton X-100, 0.5% SDS, 0.5% sodium deoxycholate, 150 mM NaCl), supplemented with 5 mM NaF, 2 mM sodium orthovanadate, and protease inhibitors (Roche, Laval, QB). The proteins (30 μ g/lane) were subjected to electrophoresis on 10% polyacrylamide gels containing SDS and transferred to nitrocellulose membranes. The membranes were blocked by incubation in 5% BSA in Tris-buffered saline pH 7.5, and 0.1% Tween-20 (TBST) and then incubated with antibodies against phospho-ERK1/2 or phosphor-MEK (titre 1:1000) (Cell Signaling), or ERK1 and ERK2 or MEK1 and MEK2 (titre 1:1000 each) (Santa Cruz Bio, Santa Cruz, CA) in 1% BSA in TBST. The blots were washed and then incubated in appropriate secondary antibody-HRP conjugates in 1% BSA in TBST. HRP was detected with ECL reagent (Pierce Chemical Co.) and exposed to ECL X-ray film (Amersham).

2.7. Quantitation of cytokines

Supernatants were collected for cytokine analysis. Cytokine levels were quantitated using ELISA kits from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. Conditioned media (100 μ l) was added to antibody-coated polystyrene wells and incubated for 2 h. After washing, the plates were incubated with biotin-labeled anti-cytokine antibody for 2 h. The plates were washed and incubated for 20 min with a streptavidin/horseradish peroxidase conjugate. The plates were washed and incubated with trimethylbenzidine and peroxide, to detect the horseradish peroxide. The reaction was stopped by the addition of 2N H₂SO₄ and the absorbance read at 540 and 450 nm on a Titertek Multiskan MCC/340 microplate reader.

3. Results

3.1. Phytochemical analysis

The phytochemical analysis (Table 1) demonstrated the presence of the characteristic alkaloids of authentic *Uncaria tomentosa* and the profile was very similar to an authentic sample in good condition analyzed previously in our laboratory (Lemaire et al., 1999).

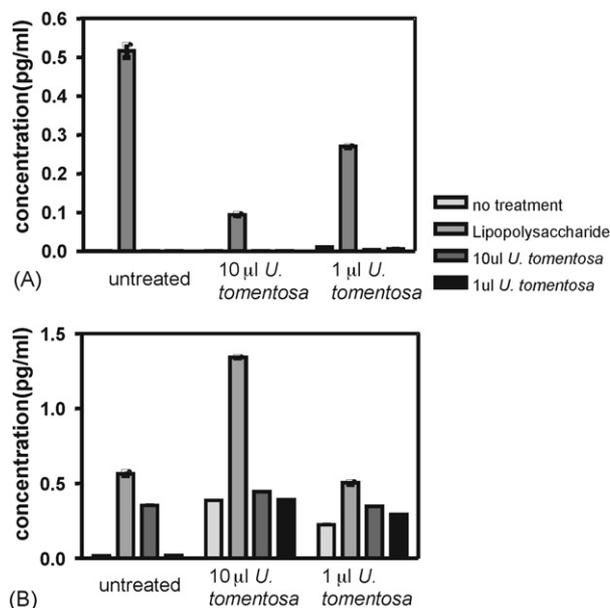


Fig. 2. Levels of TNF- α and IL-1 β secreted by THP-1 cells treated in the presence or absence of *Uncaria tomentosa* and LPS stimulation. THP-1 cells were pre-treated with culture media or 1 or 10 μ l of *Uncaria tomentosa* extract for 24 h. They were then treated with culture media, 5 μ g/ml LPS, 1 μ l, or 10 μ l of *Uncaria tomentosa* extract or left untreated for 24 h. The supernatant was collected and analyzed for the levels of secreted TNF- α (panel A) or IL-1 β (panel B) using ELISA analysis.

3.2. Effects of *Uncaria tomentosa* extracts on LPS-dependent cytokine expression

The effects of *Uncaria tomentosa* extracts on cytokine expression were determined using THP-1 monocyte-like cells treated in the presence or absence of bacterial LPS. Treatment of THP-1 cells with LPS stimulated the expression of both IL-1 β by >20-fold, and TNF- α by >5-fold (Fig. 2). A remarkable feature of these experiments is that the pre-treatment of LPS-stimulated THP-1 monocyte-like cells with *Uncaria tomentosa* extracts augmented the expression of IL-1 β by 2.4-fold while inhibiting the expression of TNF- α by 5.5-fold. It is highly unusual to see the response of these two cytokines to operate in opposite directions. The treatment of THP-1 monocytes with *Uncaria tomentosa* extracts alone augmented the expression of IL-1 β by greater than 20-fold, but did not affect the expression of TNF- α . All experiments were run in triplicate and data were analyzed in triplicate. Only statistically significant data was reported as identified by Student's *t*-test comparing treated samples to untreated samples ($P > 0.05$).

3.3. Cell viability

To determine if the effects of *Uncaria tomentosa* extracts on THP-1 cytokine secretion were related to cell death, the effects of *Uncaria tomentosa* extracts on cell viability were measured. Cell viability was determined by measuring the proportion of dead cells following staining with the vital dye trypan blue, and by determining DNA fragmentation using flow cytometry. Trypan blue staining showed that the overall death rate was less than

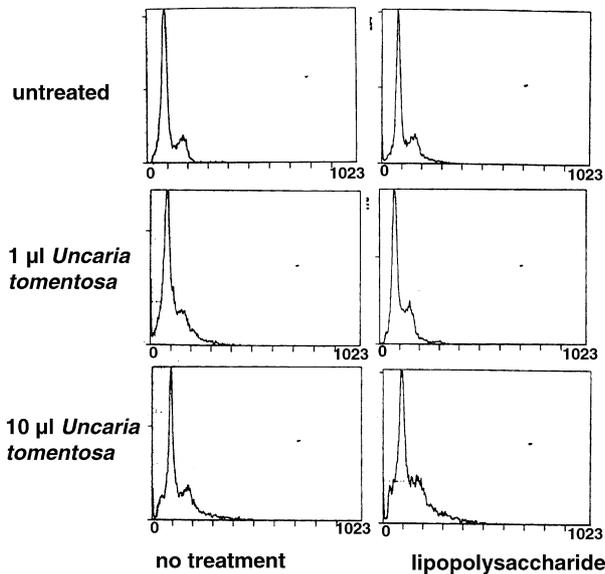


Fig. 3. Flow cytometry analysis of THP-1 cells treated in the presence or absence of *Uncaria tomentosa* and LPS stimulation. Samples were treated with culture media or 1 or 10 μl of *Uncaria tomentosa* in the presence or absence of 5 $\mu\text{g/ml}$ LPS stimulation. Following a 24 h incubation, cells were stained with propidium iodide and analyzed on a Beckman Coulter Epics flow cytometer. No significant differences in cell profiles were detected in any of the samples.

10% for THP-1 cells treated treatment with LPS or *Uncaria tomentosa* extracts (not shown). There was no increase in the proportion of dead cells following *Uncaria tomentosa* treatment. Using flow cytometry of propidium iodide-stained cells, the percentage of dead cells (cells with less than 2N DNA content) in the LPS-stimulated and untreated cells was similar to the LPS- and *Uncaria tomentosa*-treated cells (7.1% versus 5.3%, versus 7.6%, respectively) (Fig. 3). These results indicate the treatment with *Uncaria tomentosa* extracts did not significantly alter the rate of cell death. Samples from the same experiments were used in the determination of cell viability, cytokine analysis and western analysis to eliminate potential problems associated with drug deterioration or procedural differences.

3.4. Effects of *Uncaria tomentosa* extracts on the MAP kinase pathway

To determine the mechanisms underlying the effects of *Uncaria tomentosa* extracts on cytokine secretion by THP-1 cells, we examined the effects on activation of the MAP kinase pathway. Treatment of THP-1 cells with LPS promoted the phosphorylation of ERK and MEK. Using immunoblot analysis, we determined that the treatment of LPS-stimulated THP-1 cells with *Uncaria tomentosa* extracts blocked ERK phosphorylation in a dose-dependent manner (Fig. 4). At high doses, treatment with *Uncaria tomentosa* extracts promoted a complete blockage of ERK phosphorylation, whereas there was only partial blockage at lower dose treatments. However, treatment of THP-1 cells with *Uncaria tomentosa* extracts did not alter the expression of the ERK1 and ERK2 proteins. Similarly, phosphorylation of MEK, which is a protein found upstream of ERK, was also blocked in a dose-dependent manner, showing a complete inhi-

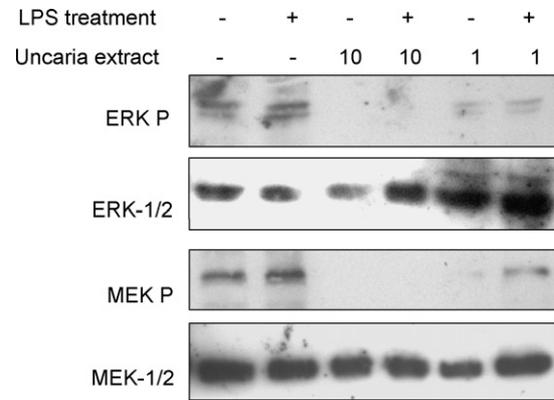


Fig. 4. Immunoblot analysis performed on protein extracts from THP-1 cells treated in the presence or absence of *Uncaria tomentosa* and LPS stimulation. Western analysis was performed on lysates from cells that were treated with culture media or 1 or 10 μl of *Uncaria tomentosa*, in the presence or absence of 5 $\mu\text{g/ml}$ LPS. Thirty micrograms of protein was loaded per lane. ERK and MEK phosphorylation were detected using phospho-specific antibodies. MEK1/2 demonstrates equivalent loading of proteins across samples. The total amount of ERK and MEK protein found in the cell was detected with anti-MEK1/2 and anti-ERK1/2.

tion of phosphorylation at high dose treatment and only partial inhibition at lower dose treatments. Treatment of THP-1 cells with *Uncaria tomentosa* extracts did not alter the expression of the MEK1 and MEK 2 proteins (Fig. 4).

4. Discussion

These experiments have demonstrated a significant cellular response elicited by the treatment of THP-1 cells with *Uncaria tomentosa* extracts. The mechanism of action of *Uncaria tomentosa* appears to involve a link between the expression of the cytokines, TNF- α , and IL-1 β , and the activation of the MAP kinase-signaling pathway. In these studies, ERK phosphorylation and the secretion of TNF- α and IL-1 β were activated by treatment of THP-1 monocyte-like cells with bacterial LPS. However, the most interesting observation made during these studies was that treatment of LPS-activated THP-1 cells with *Uncaria tomentosa* extracts had different effects on TNF- α and IL-1 β expression: TNF- α expression was inhibited while IL-1 β expression was enhanced. Further, treatment with *Uncaria tomentosa* extracts blocked ERK phosphorylation in a dose-dependent manner. The ability of *Uncaria tomentosa* extracts to differentially regulate TNF- α and IL-1 β expression is puzzling since TNF- α and IL-1 β are regulated by similar promoter elements and have been shown to respond similarly to stimuli. TNF- α and IL-1 β are very well characterized and extensively studied genes that are primarily regulated at the level of transcription (Muller et al., 1993; Adcock, 1997). Our current results suggest that *Uncaria tomentosa* can alter some unknown element that can distinguish their expression. This could be clinically relevant since specific blockage of TNF- α signaling (usually using antibody-based therapies) has been used to treat chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease (Nakar et al., 2003; Baker, 2004). Understanding the exact mechanism by which *Uncaria tomentosa* is able to control

independent regulators of the cellular immune response could be key in the development of novel chemotherapeutic agents or development of anti-inflammatory treatments.

In these studies, we showed that treatment of THP-1 cells with LPS activated ERK phosphorylation and increased the secretion of TNF- α and IL-1 β consistent with a number of published observations. It has been shown that LPS-dependent activation of TNF- α and IL-1 β is partially dependent on the activation of the MAP kinase pathway and that LPS stimulation strongly activates both MAP kinase phosphorylation and TNF- α and IL-1 β protein expression (Willis and Nisen, 1996; Scherle et al., 1998; Carter et al., 1999; van der Bruggen et al., 1999; Chakravorty et al., 2001; Rutault et al., 2001; Setty and Sigal, 2005). The data presented in this report indicate that treatment of THP-1 cells with LPS in the presence of *Uncaria tomentosa* extracts alters the expression of proinflammatory cytokines while blocking the MAP kinase signaling pathway in a dose-dependent manner. Various inhibitors, including RO 09-2210 (a MAP kinase inhibitor), PD 098059 (a MEK inhibitor), and UO126 (a MEK inhibitor) have demonstrated that the Raf-1/MEK1-MEK2/ERK1-ERK2 signaling pathway is involved in the expression of the TNF- α and IL-1 β proteins (Scherle et al., 1998; Carter et al., 1999; van der Bruggen et al., 1999; Chakravorty et al., 2001; Rutault et al., 2001). In addition, treatment with okadaic acid, a potent stimulator of TNF- α and IL-1 β protein production, enhanced phosphorylation of ERK1/ERK2, enhanced the kinase activity of JNK, and increased the binding activity of NF- κ B (Tuyt et al., 1999). This suggests a common factor between the expression of IL-1 β and TNF- α and activation of the MAP kinase pathway may be the transcription factor NF- κ B (Fig. 5).

Several studies have suggested that the activities associated with *Uncaria tomentosa* extracts might be mediated by effects on NF- κ B. For example, NF- κ B is involved in the activation of the promoters that regulate the expression of TNF- α , IL-1, IL-2, IL-6, and IL-8 and LPS-dependent activation of cytokine secretion is associated with NF- κ B activation and MAP kinase phosphorylation (Muller et al., 1993; Adcock, 1997; Rutault et al., 2001).

NF- κ B is also involved in lymphocyte activation and proliferation and can regulate apoptosis by controlling the expression of anti-apoptotic genes (Sandoval-Chacon et al., 1998; Sandoval et al., 2000; Akesson et al., 2003b). NF- κ B is also involved in the ability of *Uncaria tomentosa* extracts to enhance the ability of cells to self-repair both single strand and double strand breaks (which are usually lethal to the cell) (Sandoval et al., 2000; Sheng et al., 2000b; Akesson et al., 2003a,b; Setty and Sigal, 2005; Mammone et al., 2006). However, the results of our studies suggest that the mechanisms of action of *Uncaria tomentosa* extracts are more complicated than just altering NF- κ B. Simply inhibiting NF- κ B would be expected to inhibit both TNF- α and IL-1 β expression, while in our experiments, only TNF- α expression was inhibited while IL-1 β expression was enhanced. This suggests that *Uncaria tomentosa* extracts alter some unknown element that can distinguish TNF- α and IL-1 β expression and experiments to elucidate these mechanisms are in progress.

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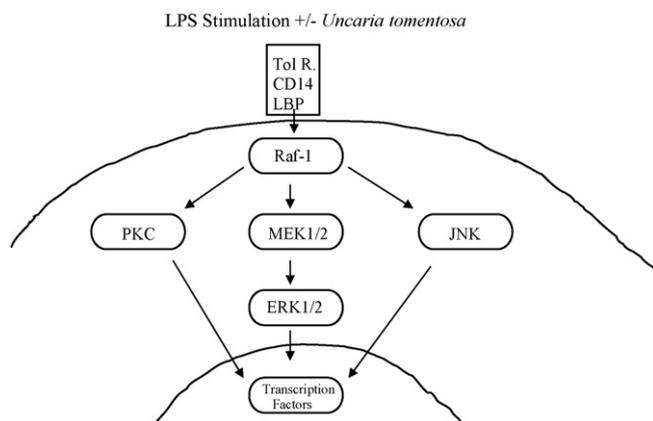


Fig. 5. Proposed signaling pathway for LPS stimulation of THP-1 cells treated with *Uncaria tomentosa* extracts resulting in variable expression of proinflammatory cytokines, including TNF- α , IL-1 β , and NF- κ B transcription factor precursors (Tol R, toll-like receptors; CD14, surface receptors; LBP, lipopolysaccharide binding protein).

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