Journal of Ethnopharmacology 127 (2010) 685–693

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Uncaria tomentosa acts as a potent TNF-α inhibitor through NF-κB

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ARTICLE INFO

Article history:
Received 20 September 2009
Received in revised form 1 December 2009
Accepted 1 December 2009
Available online 6 December 2009

Keywords:
Uncaria tomentosa
Cytokine
Inflammation
NF-κB
TNF-α

ABSTRACT

Aim of the study: Uncaria tomentosa, commonly known as Cat's Claw or *Uña de gato*, is a medicinal plant that has been shown to have effective anti-inflammatory activities. We have previously shown that treatment of monocyte-like THP-1 cells with *Uncaria tomentosa* inhibits the production of the pro-inflammatory cytokine TNF-α while augmenting the production of IL-1β. Since TNF-α and IL-1β are usually regulated similarly and share a number of common promoter elements, including NF-κB and AP-1, the ability of *Uncaria tomentosa* to differentially regulate these inflammatory cytokines is of particular interest.
Materials and methods: To determine the mechanism of action of *Uncaria tomentosa,* we investigated the effects of specific inhibitors of NF-κB on cellular responses including transcription factor activation using TransAM assays, the expression of cytokines as measured by ELISA, and cell survival as measured by changes in cell number following treatment.
Results: Treatment with *Uncaria tomentosa* inhibited the LPS-dependent activation of specific NF-κB and AP-1 components. In addition, treatment with *Uncaria tomentosa* enhanced cell death when NF-κB was inhibited. The ability of *Uncaria tomentosa* to inhibit TNF-α production was diminished when NF-κB activation was prevented by drugs that mask NF-κB subunit nuclear localization signals, while IL-1β expression was unchanged.
Conclusions: These results demonstrate that *Uncaria tomentosa* is able to elicit a response via an NF-κB-dependent mechanism. Further studies to characterize the mechanism by which *Uncaria tomentosa* can affect this pathway could provide a means to develop anti-TNF-α therapies.

1. Introduction

Uncaria tomentosa (Willd.) DC (Rubiaceae), or Uña de Gato, commonly known as Cats claw, is a medicinal plant native to the Peruvian Amazon. It has traditionally been used by Ashaninka Indians to treat disorders including arthritis, heart disease, cancer, and other inflammatory diseases (Piscoya et al., 2001; Heitzman et al., 2005; Cheng et al., 2007). The woody vines are typically prepared in a ground tea-like preparation and served as a hot water concoction (Pilarski et al., 2007). Attempts to isolate the medically relevant properties of *Uncaria tomentosa* have identified powerful antioxidant, anti-viral, and anti-mutagenic properties (Goncalves et al., 2005; Reis et al., 2008). *Uncaria tomentosa* is also able to inhibit the secretion of the pro-inflammatory cytokine, TNF-α (Sandoval et al., 2000, 2002; Allen-Hall et al., 2007). Anti-TNF-α therapy, using specific antibodies, is currently the most effective treatment for autoimmune disorders including rheumatoid arthritis although its general usefulness is limited by cost (Handel et al., 2000; Bright, 2007). Therefore, utilizing *Uncaria tomentosa* as a potential inhibitor of TNF-α secretion is of possible clinical value.

Treatment of THP-1 cells with a pro-inflammatory agent, such as the bacterial endotoxin, LPS, leads to enhanced secretion of, TNF-α and IL-1β (Zhang and Ghosh, 2000). Both cytokines are expressed similarly. In addition, TNF-α and IL-1β are able to work synergistically to enhance physiological responses (Granet et al., 2004). However, we previously showed that treatment with *Uncaria tomentosa* inhibits TNF-α expression but augments IL-1β (Allen-Hall et al., 2007). The expression of TNF-α and Il-1β is regulated by the NF-κB and AP-1 transcription factors, which in turn can be activated by TNF-α and Il-1β to potentiate an inflammatory response (Granet et al., 2004). To our knowledge, *Uncaria tomentosa* is the first compound that can differentially regulate these two cytokines. The ability of *Uncaria tomentosa* treatment to inhibit TNF-α but allow IL-1β secretion may allow appropriate activation of the immune system without promoting the synergistic responses.

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doi:10.1016/j.jep.2009.12.004

The nuclear factor kappa B (NF-κB) family of closely related, highly regulated transcription factors includes the p50, p65, p52, Rel B, and cRel subunits. Each subunit contains a rel homology domain and can form homo- or heterodimers following activation. Inactive NF-κB is bound to the inhibitory protein, IκB, blocking its nuclear localization signal and causing NF-κB to be sequestered in the cytoplasm. Following activation, IκB is proteolytically degraded and the NF-κB subunits are released to enter the nucleus where they can bind DNA at κB consensus sites to promote gene transcription (Zhang et al., 2007; Ortiz-Lazareno et al., 2008). NF-κB activation is involved in the inflammatory response as well as in the regulation of cellular apoptosis, or programmed cell death. In 'normal' cells, activation of NF-κB generally promotes apoptosis, however, in malignant cells, activation of NF-κB can promote cell survival and the inhibition of NF-κB can decrease tumor growth (Paur et al., 2008). In the absence of NF-κB activation, treatment with TNF-α promotes apoptosis in most cells but enhances the survival of the leukocytes involved in the immune response (Hacker and Karin, 2002; Amit and Ben-Neriah, 2003; Walmsley et al., 2005).

Similarly, the activator protein-1 (AP-1) family of transcription factors are important mediators of the inflammatory response and are activated by bacterial LPS, cytokines, and growth factors. Activation of AP-1 contributes to rheumatoid arthritis by promoting an inflammatory response and joint erosion (Hou et al., 2007; Gopalakrishnan and Tony Kong, 2008; Shiozawa and Tsumiyama, 2009). The AP-1 family consists of highly regulated subunits including different Jun and Fos proteins. Jun family members include c-Jun, JunB, and JunD which can form stable homodimers or heterodimers with the Fos family members that include c-Fos, FosB, Fra-1, and Fra-2. Activation of survival pathways, such as the MAP kinase-signaling pathway, can also activate AP-1 activity. The Jun subunit homodimer can bind to the Fas promoter and activate the intrinsic apoptotic pathway while binding of the Fos subunit to Jun (to create the Fos-Jun heterodimer) can inactivate Jun-mediated Fas-induced cell death (Ahmed-Choudhury et al., 2003; Gopalakrishnan and Tony Kong, 2008). Therefore, the pathway stimulated following AP-1 activation depends on the relative proportion of homodimers versus heterodimers formed (Nakamura et al., 1991).

In this study we investigated the mechanism utilized by *Uncaria tomentosa* to differentially regulate the secretion of the pro-inflammatory cytokines TNF-α and IL-1β and to regulate cell survival. We determined that while *Uncaria tomentosa* inhibited LPS-dependent activation of all of the AP-1 family members it differentially regulated activation of the NF-κB family members. This suggests that *Uncaria tomentosa* acts via modulation of NF-κB. By using specific NF-κB inhibitors, we were able to show that NF-κB mediated the differential secretion of TNF-α and IL-1β resulting from treatment with *Uncaria tomentosa*. We also showed that treatment with *Uncaria tomentosa* in cells where NF-κB was inhibited significantly enhanced cell death, suggesting a role for *Uncaria tomentosa* in the treatment of various diseases including cancer.

2. Materials and methods

2.1. Cell culture and treatments

THP-1 cells, obtained from the American Type Culture Collection (ATCC Manassas, VA), were maintained in RPMI 1640 media supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotic/antimycotic solution (Invitrogen, Burlington, ON) in a 5% CO_2 supplemented, 37 °C environment. For experiments, the cells were collected by centrifugation at 400 × *g* and resuspended at 2×10^6 cells/ml in serum-free media (unless otherwise specified). The bark from wild *Uncaria tomentosa* plants was dried, ground, and extracted by exhaustive percolation in 95% ethanol, as described (Allen-Hall et al., 2007). Two different preparations of *Uncaria tomentosa* were used and compared by HPLC to ensure equal quantities of marker components were present (treatment with different concentration of the extracts were required to equalize the marker components and achieve similar responses). Immediately prior to cell treatment, the ethanol-extracted *Uncaria tomentosa* was resuspended in 1:4 ethanol:RPMI serum-free media and appropriate doses of this stock solution were applied to the cell cultures as indicated. For some experiments cells were treated with *Uncaria tomentosa* in the presence or absence of the NF-κB inhibitors, 100 μg/ml SN50 or MSN50 (Cedarlane Technology, Mississauga, ON) or 10 μg/ml of AZT (Sigma–Aldrich). For each experiment, ethanol was used at the same concentration as a negative control.

2.2. Quantitation of cytokines

Cells in serum-free media were treated with the indicated concentrations of *Uncaria tomentosa*, in the presence or absence of 2.5 μg/ml LPS (*E. coli* serotype 0127 Sigma–Aldrich, St. Louis, MO) for 24 h. Conditioned media was collected and the cytokine levels were measured using ELISA kits from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. Briefly, conditioned media (100 μl) was added to antibody-coated polystyrene wells and incubated for 2 h. After washing, the plates were incubated with a biotin-labeled anti-cytokine antibody for 2 h. The plates were washed and incubated for 20 min with a streptavidin/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 H_2SO_4 and the absorbance was read at 540 and 450 nm using a Titertek Multiskan MCC/340 microplate reader. The experiments were performed in triplicate and the results were analyzed using a Students' *t*-test of three independent experiments.

2.3. Trans AM kit NF-κB and AP-1

The activation of the AP-1 and NF-κB transcription factor subunits was determined using Trans AM kits according to manufacture's instructions (Active Motif, Carlsbad, CA). Cells in serum-free media were treated with the indicated concentrations of *Uncaria tomentosa*, in the presence or absence of 2.5 μg/ml LPS (*E. coli* serotype 0127) for 24 h. Nuclear extracts were prepared in hypotonic buffer and lysed with 0.5% Nonident P-40. The nuclear pellet was collected and resuspended in complete lysis buffer (Trans AM kit) and the protein concentration was determined using a BCA assay (BioRad, Mississauga, ON). The concentration of the activated transcription factor was detected using an ELISA-based method. The nuclear extract was incubated for 1 h with a plate coated with an oligonucleotide that corresponded to a transcription factor consensus site. The wells were washed and incubated with an antibody against the indicated transcription factor subunit. An anti-IgG HRP conjugate was added and then the developing solution was added and the colorimetric reaction was measured using a Titertek Multiskan MCC/340 microplate reader at 450 nm. The experiment was performed in triplicate and the results were analyzed using a Students' *t*-test of three independent experiments.

2.4. MTT assay

The THP-1 cells were resuspended in culture media at a concentration of 1×10^5 cells/ml and treated with 0, 40, 160 μg/ml or 320 μg/ml of *Uncaria tomentosa* in the presence or absence of 2.5 μg/ml LPS (*E. coli* serotype 0127). For some experiments the cells were treated with *Uncaria tomentosa* in the presence

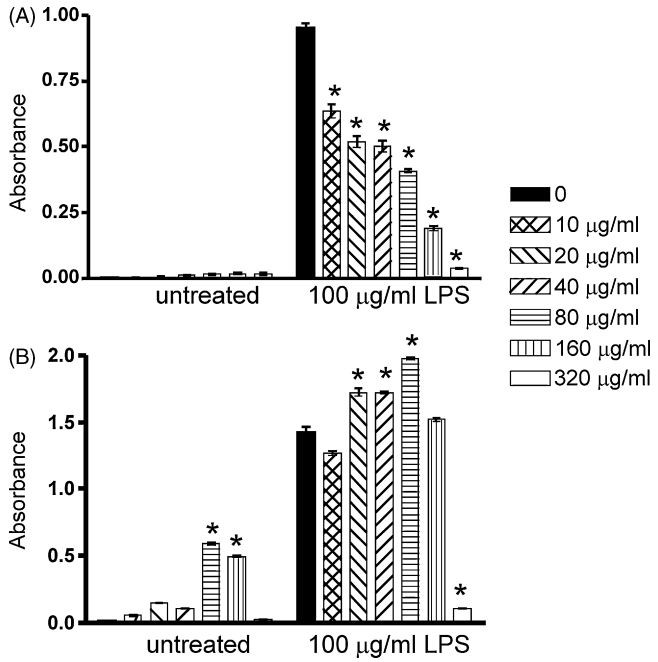

Fig. 1. Levels of TNF-α and IL-1β cytokine secreted by THP-1 cells treated in the presence or absence of *Uncaria tomentosa* and LPS. THP-1 cells were treated with increasing concentrations of *Uncaria tomentosa* as indicated followed by stimulation with 0 or 2.5 μg/ml LPS. Following 24 h incubation, the supernatant was collected and analyzed for the level of secreted TNF-α (panel A) or IL-1β (panel B) using ELISA. Statistical analysis was performed using a Students' *t*-test, error bars are reported as standard error of the mean for the experiment performed in quadruplicate. Asterisk (*) indicates a significant difference ($p < 0.05$) from control levels for cells treated with *Uncaria tomentosa*.

or absence of the NF-κB inhibitors, 100 μg/ml SN50 or MSN50 (Cedarlane Technology, Mississauga, ON) or 10 μg/ml of AZT (Sigma–Aldrich). Cells were plated on 96-well plates, in quintuplicate, at 10^3 cells/well and the number of cells determined each day. On each day of culture, 5 pg of thiazolyl blue terazolium bromide (Sigma–Aldrich, M2128) was added to the sample and control wells and incubated at 37 °C and 5% CO_2 for 4 h. Then, 200 μl/well of DMSO was added to dissolve the converted crystals. The absorbance was read at 540 nm using a Titertek Multiskan MCC/340 and the trend for each day plotted to determine the relative growth in response to each treatment. Significant differences in the values between treatment conditions were determined by using a one-way ANOVA.

3. Results

3.1. Cytokine secretion by Uncaria tomentosa-treated THP-1 cells

THP-1 cells were treated with increasing concentrations of *Uncaria tomentosa* extract in the presence or absence of 2.5 μg/ml LPS for 24 h. The levels of the TNF-α and IL-1β cytokines in the conditioned media were determined. Treatment of THP-1 cells with LPS significantly ($p < 0.01$) increased the secreted levels of both TNF-α and IL-1β (Fig. 1). Treatment with *Uncaria tomentosa* inhibited the secretion of TNF-α in LPS-treated cells in a dose-dependent manner: 10 μg/ml *Uncaria tomentosa* extract inhibited TNF-α secretion by 33%; 40 μg/ml inhibited by 45%; 160 μg/ml inhibited by 80%; and 320 μg/ml inhibited TNF-α secretion by 95%. In contrast, treatment with *Uncaria tomentosa* enhanced LPS-dependent expression of IL-1β: 40 μg/ml enhanced IL-1β by 1.2-fold and 160 μg/ml enhanced IL-1β by 1.4-fold, although 320 μg/ml *Uncaria tomentosa* completely blocked LPS-dependent secretion of IL-1β. These results demonstrate that treatment with *Uncaria tomentosa* differentially regulated the expression of IL-1β and TNF-α by LPS-stimulated THP-1 cells. Extremely high doses of *Uncaria tomentosa* (320 μg/ml) produced variable effects and therefore a high dose of 160 μg/ml was used in further experiments. A low dose of 40 μg/ml was selected for further experiments.

3.2. Active NF-κB and AP-1 transcription factors in Uncaria tomentosa-treated cells

The relative amount of each active transcription factor family member was determined using the TransAM Active Motif NF-κB or AP-1 family kits. THP-1 cells were treated with 0, 40, or 160 μg/ml *Uncaria tomentosa* in the presence or absence of 2.5 μg/ml LPS for 24 h and then the amount of activated transcription factor was determined. The amount of active c-Jun was increased 2.2-fold ($p < 0.01$) and the amount of JunB was increased 1.6-fold following treatment with LPS (Fig. 2). Treatment with 160 μg/ml *Uncaria tomentosa* inhibited c-Jun activity by 66% ($p < 0.001$) and JunB activity by 60% ($p < 0.05$) in LPS-treated cells. However, treatment of cells with *Uncaria tomentosa* in the absence of LPS did not alter c-Jun activity and only inhibited JunB activity by 32% ($p < 0.01$). Treatment of THP-1 cells with LPS did not activate FosB, JunD, Fra-1, Fra-2, or c-Fos. Inclusion of 160 μg/ml *Uncaria tomentosa* inhibited the activation of all of the AP-1 subunits in both the presence and absence of LPS treatment. For example, treatment with 160 μg/ml *Uncaria tomentosa*, inhibited c-fos activity in LPS-treated cells by 81% ($p < 0.01$). FosB activity was decreased following treatment with 160 μg/ml *Uncaria tomentosa* by 62% ($p < 0.05$) in untreated cells and by 48% ($p < 0.05$) in LPS-treated cells. Fra-1 activity was decreased 68% in the presence of 160 μg/ml *Uncaria tomentosa* ($p < 0.05$).

The amount of all of the activated NF-κB family members was significantly increased by treatment with LPS. Activated p52 was increased by 1.4-fold, p65 by 3.2-fold ($p < 0.05$), p50 by 5.7-fold, and RelB by 270-fold ($p < 0.01$) (Fig. 3). However, treatment with *Uncaria tomentosa* had differential effects on the levels of activated NF-κB subunits. Treatment with both LPS and high dose *Uncaria tomentosa* synergistically increased the level of activated p52 by 4.6-fold ($p < 0.01$), while high dose *Uncaria tomentosa* inhibited the LPS-dependent activation of the p65 (29%), RelB (96%), and p50 (41%) subunits ($p < 0.05$). *Uncaria tomentosa* treatment did not inhibit the amount of activated NF-κB subunits in the absence of LPS.

3.3. Effect of NF-κB inhibitors on cytokine secretion by Uncaria tomentosa-treated cells

To determine the role of NF-κB in mediating *Uncaria tomentosa* activity, TNF-α and IL-1β secretion was measured in THP-1 cells treated with *Uncaria tomentosa* in the presence or absence of LPS and in the presence or absence of the NF-κB inhibitors, SN50 or AZT.

SN50 inhibits NF-κB translocation into the nucleus, a late event in the pathway (100 μg/ml SN50 can inhibit NF-κB by 90% [Lin et al., 1995]). Treatment of THP-1 cells with SN50 inhibited the amount of TNF-α secreted by LPS-stimulated THP-1 cells by 79% compared to untreated cells or cells treated with the negative control peptide, MSN50 (Fig. 4). Treatment with 160 μg/ml *Uncaria tomentosa* inhibited LPS-dependent secretion of TNF-α by 85% ($p < 0.01$). However, in the cells treated with both LPS and *Uncaria tomentosa,* SN50 resulted in a small increase in TNF-α secretion compared to control cells. Neither *Uncaria tomentosa* nor SN50 altered the level of TNF-α secreted by THP-1 cells in the absence of LPS. In contrast, SN50 had different effects on IL-1β secretion. THP-1 cells treated only with *Uncaria tomentosa* enhanced IL-1β secretion and this was inhibited in the presence of both SN50 and the MSN50 control peptide. Treatment of THP-1 cells with LPS also increased the level of IL-1β,

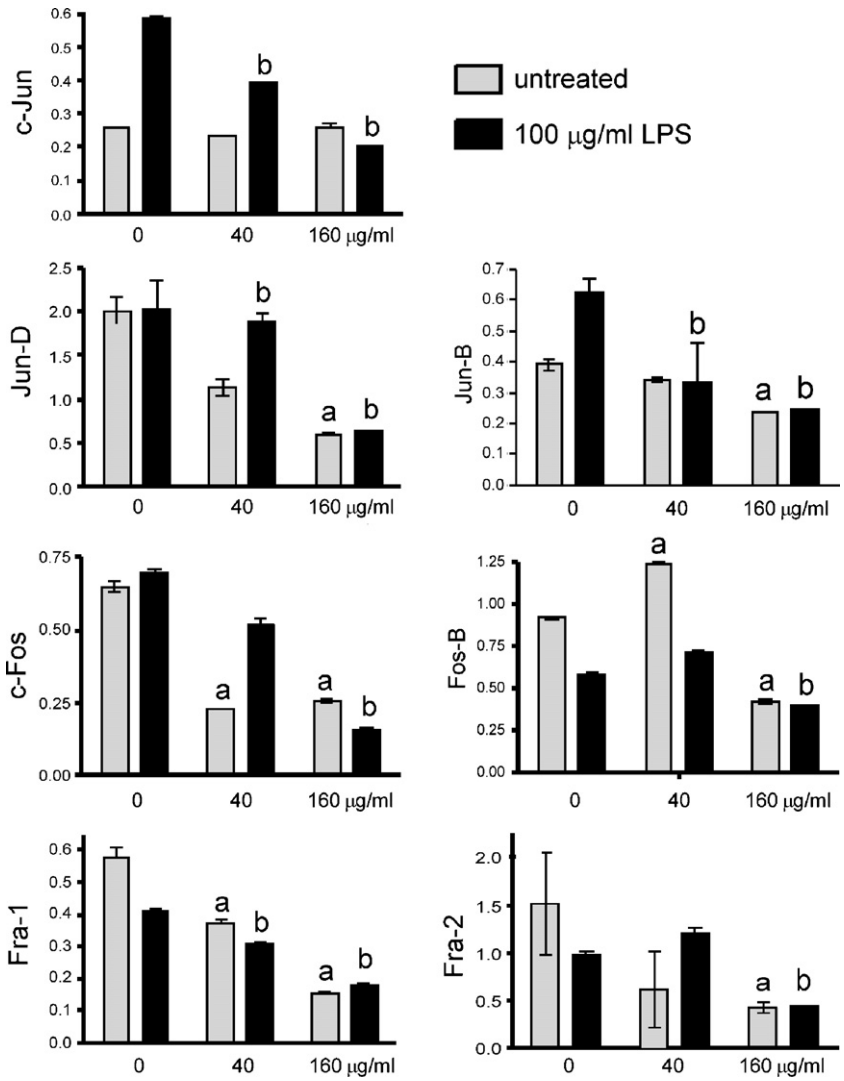

Fig. 2. Relative amount of activated AP-1 family members following treatment with *Uncaria tomentosa* and LPS. THP-1 cells were treated with 0, 40, or 160 μg/ml *Uncaria tomentosa* in the presence or absence of 2.5 μg/ml LPS. Following 24 h incubation, the nuclear extract was collected. The level of active AP-1 family members were determined using the Trans AM AP-1 kit according to the manufacturer's instructions, and reported as an absorbance level of the colorimetric substrate. Statistical analysis was performed using a Students' *t*-test, error bars are reported as standard error of the mean for the experiment performed in quadruplicate. 'a' indicates a significant difference ($p < 0.05$) from the control levels in cells treated with *Uncaria tomentosa*. 'b' indicates a significant difference ($p < 0.05$) from control cells in cells treated with LPS.

however the addition of *Uncaria tomentosa* or SN50 had only small effects on IL-1β secretion (within 10% variance of the untreated control).

In contrast with SN50, treatment with the NF-κB inhibitor AZT had very little effect on either TNF-α and IL-1β secretion. AZT inhibits NF-κB activation (in particular the p65/p50 heterodimer) relatively early in the activation pathway by blocking IκB phosphorylation (Ghosh et al., 2003). Treatment of THP-1 cells with AZT caused a small increase in TNF-α secretion (Fig. 5). As shown previously, treatment with LPS strongly increased TNF-α secretion ($p < 0.01$) and this was almost completely blocked by the addition of 160 μg/ml *Uncaria tomentosa*. However, the addition of AZT did not significantly alter TNF-α secretion by these cells. The secretion of IL-1β was enhanced by treatment of THP-1 cells with *Uncaria tomentosa* in the presence and absence of LPS. IL-1β secretion in LPS-treated cells was further enhanced 2.2-fold by 160 μg/ml *Uncaria tomentosa* ($p < 0.05$). Inclusion of AZT promoted a small increase in the level of IL-1β secreted by LPS-treated cells in the absence or presence of 40 μg/ml *Uncaria tomen*tosa although AZT did decrease IL-1β secretion in LPS-treated cells treated with 160 μg/ml *Uncaria tomentosa.*

3.4. Effect of Uncaria tomentosa on THP-1 cell proliferation

The role of NF-κB and *Uncaria tomentosa* on THP-1 cell proliferation and viability was determined using MTT assays in cells treated with different doses (0, 40 or 160 μg/ml) of *Uncaria tomentosa*, LPS, and/or NF-κB inhibitors (SN50 and AZT). Cell viability was determined each day and growth curves plotted. Treatment with *Uncaria tomentosa* alone did not reduce cell viability at any of the tested concentrations (Fig. 6). [There were some variations in cell viability at the highest doses of *Uncaria tomentosa* between different trials, however the experiment in Fig. 6 shows representative data from at least three individual experiments.] Inclusion of the NF-κB inhibitors, SN50 or AZT, significantly decreased the viability of THP-1 cells treated with *Uncaria tomentosa* but had relatively small effects on the viability of untreated or LPS-treated cells in the absence of *Uncaria tomentosa*. Treatment with AZT did inhibit cell proliferation by approximately 30%.

Treatment of THP-1 cells with both the NF-κB inhibitor SN50 and 320 μg/ml *Uncaria tomentosa* almost completely blocked cell proliferation ($p < 0.05$). The addition of LPS decreased the concentration of *Uncaria tomentosa* that was required to completely inhibit

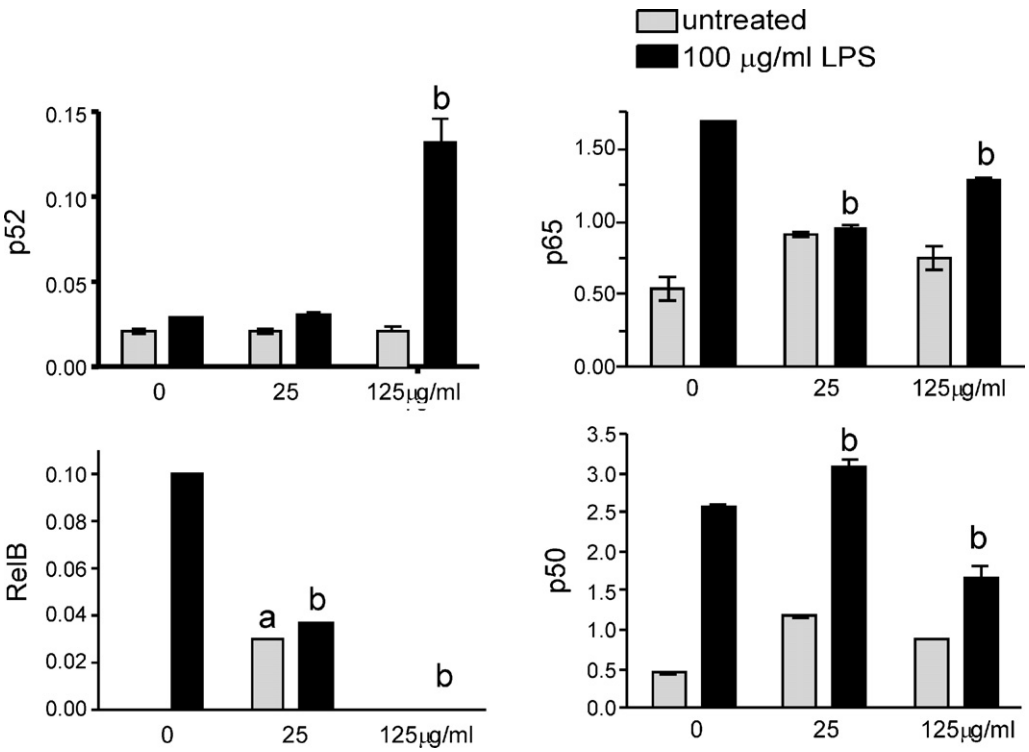

Fig. 3. Relative amount of activated NF-κB family members following treatment with *Uncaria tomentosa* and LPS. THP-1 cells were treated with 0, 40, or 160 μg/ml *Uncaria tomentosa* in the presence or absence of 2.5 μg/ml LPS. Following a 24 h incubation, the nuclear extract was collected. The levels of active NF-κB family members were determined using the Trans AM NF-κB kit according to the manufacturer's instructions and reported as an absorbance level of the colorimetric substrate. Statistical analysis was performed using a Students' *t*-test, error bars are reported as standard error of the mean for the experiment performed in quadruplicate. 'a' indicates a significant difference ($p < 0.05$) from the control levels in cells treated with *Uncaria tomentosa*. 'b' indicates a significant difference ($p < 0.05$) from control cells in cells treated with LPS.

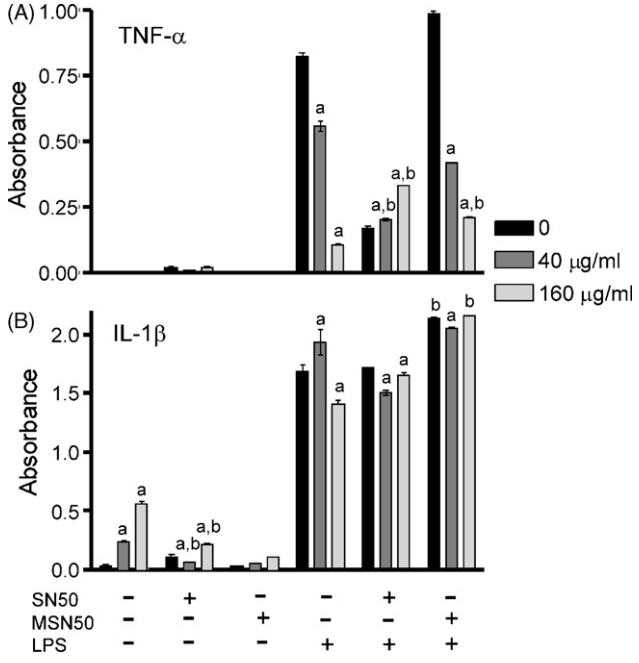

Fig. 4. Levels of TNF-α and IL-1β secreted by THP-1 cells treated with *Uncaria tomentosa,* the NF-κB inhibitor SN50, and LPS. THP-1 cells were treated with 0, 40, or 160 μg/ml *Uncaria tomentosa*, 0 or 100 ng/ml SN50 (or the control MSN50 control peptide). Following 24 h incubation cells were treated with 0 or 2.5 μg/ml LPS and incubated 2 h. The supernatant was collected and analyzed for the level of secreted TNF-α (panel A) or IL-1β (panel B) by ELISA and reported as an absorbance level of the colorimetric substrate. Statistical analysis was performed using a Students' *t*-test, error bars are reported as standard error of the mean for the experiment performed in quadruplicate. 'a' indicates a significant difference ($p < 0.05$) from the control levels in cells treated with *Uncaria tomentosa*. 'b' indicates a significant difference ($p < 0.05$) from control cells in cells treated with SN50 or MSN50.

cell proliferation ($p < 0.05$). Similarly, treatment with the NF-κB inhibitor AZT and 160 or 320 μg/ml *Uncaria tomentosa* almost completely inhibited ($p < 0.01$) cell proliferation which was enhanced by the further addition of LPS.

4. Discussion

Uncaria tomentosa is a commonly used medicinal plant that has been shown to have anti-inflammatory effects. Some studies have suggested that *Uncaria tomentosa* may be useful in cancer treatment and may enhance survival following conventional cancer therapy (Cheng et al., 2007; Garcia Prado et al., 2007; Pilarski et al., 2007). While there is good evidence to support some of these claims, the mechanism of action of *Uncaria tomentosa* remains largely uninvestigated. In these experiments, we were able to show that *Uncaria tomentosa* differentially regulated the pro-inflammatory cytokines IL-1β and TNF-α and moderated cell proliferation through regulation of the NF-κB transcription factor.

Treatment of THP-1 cells with *Uncaria tomentosa* inhibited TNF-α expression following LPS treatment in a dose-dependent manner. At low doses of *Uncaria tomentosa*, TNF-α secretion was inhibited by 25% but at higher doses inhibition was 75%. In contrast, similar doses of *Uncaria tomentosa* augmented IL-1β secretion by 25%. To ensure that these results were not related to alterations in cell viability, MTT assays to measure cell proliferation were performed. These studies showed that the highest doses of *Uncaria tomentosa* caused a slight lag in cell growth in some experiments (but not in others). The effects at the highest doses of *Uncaria tomentosa* (320 μg/ml) were more variable than at lower doses, which might be explained by the complexity of the plant extract as well as the observation that the extraction method may affect different cellular responses (Setty and Sigal, 2005; Pilarski et al., 2007). This highlights the need

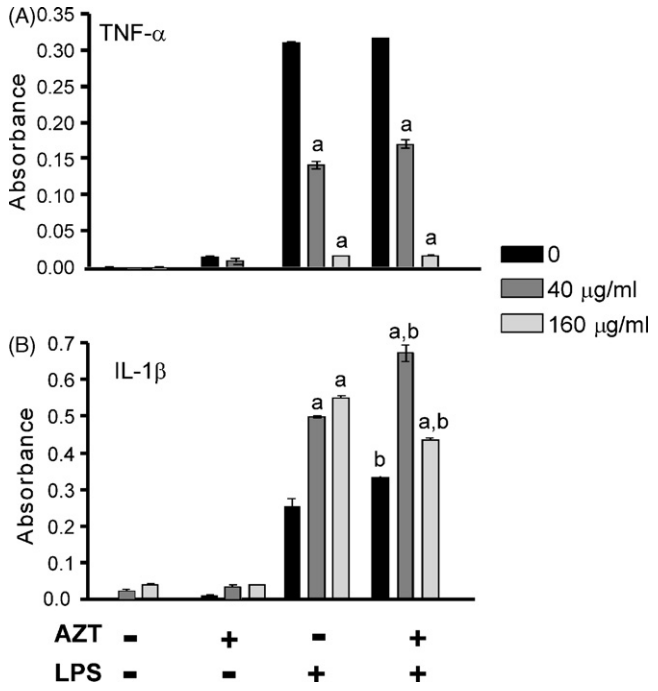

Fig. 5. Levels of TNF-α and IL-1β secreted by THP-1 cells treated with *Uncaria tomentosa,* the NF-κB inhibitor AZT, and LPS. THP-1 cells were treated with 0, 40, or 160 μg/ml *Uncaria tomentosa*, 0 or 10 μg/ml AZT, followed by treatment with 0 or 2.5 μg/ml LPS. Following 24 h incubation, the supernatant was collected and analyzed for the level of secreted TNF-α (panel A) or IL-1β (panel B) by ELISA and reported as an absorbance level of the colorimetric substrate. Statistical analysis was performed using a Students' *t*-test, error bars are reported as standard error of the mean for the experiment performed in quadruplicate. 'a' indicates a significant difference ($p < 0.05$) from the control levels in cells treated with *Uncaria tomentosa*. 'b' indicates a significant difference ($p < 0.05$) from control cells in cells treated with AZT.

for complete characterization and consistent extraction procedures.

The expression of TNF-α and IL-1β is largely controlled at the level of transcription via activation of the multiple AP-1 and NF-κB elements within the promoters both genes (Lee et al., 2006, 2008a). The expression of TNF-α and IL-1β and the activation of AP-1 and NF-κB are all enhanced by treatment with LPS or pro-inflammatory cytokines, such as IL-1β and TNF-α (Lee et al., 2008b). Treatment with *Uncaria tomentosa* was shown to activate various AP-1 and NF-κB transcription factor subunits. These results showed that: (1) treatment with LPS-activated c-Jun, JunB, p65, RelB and p50; (2) treatment with *Uncaria tomentosa* inhibited LPS-dependent activation of c-Jun, JunB, p65, RelB, and p50; (3) treatment with *Uncaria tomentosa* inhibited the activation of JunB, FosB, JunD, Fra-1, Fra-2, and c-Fos in both untreated and LPS-treated THP-1 cells; (4) in contrast, treatment with both *Uncaria tomentosa* and LPS significantly activated p52; and (5) the ability of *Uncaria tomentosa* to inhibit LPS-dependent activation of AP-1 is similar to the effect of *Uncaria tomentosa* on TNF-α secretion. Therefore, these results indicate that treatment with *Uncaria tomentosa* can inhibit activation of all AP-1 transcription factor subunits and inhibit TNF-α secretion, and that *Uncaria tomentosa* treatment only inhibits some of the NF-κB subunits but can activate p52 and enhance IL-1β secretion.

NF-κB is a promising target for the development of drugs that modulate the immune system and for anti-cancer therapy. Inhibition of NF-κB, in combination with DNA damaging anti-cancer drugs, enhances cell death and promotes patient survival (Zhang et al., 2007; Paur et al., 2008). NF-κB can mediate different activities depending on the relative expression of the various subunits and on the formation of specific dimers. The p65/p50 heterodimer is generally the most prevalent combination (Pickering et al., 2007). High concentrations of p65 are usually associated with enhanced cell survival and the p50 subunit is not required for TNF-α-enhanced cell proliferation (Mittal et al., 2006). In the absence of p50, the presence of p52 in the p65/p52 complex can mediate increased cell proliferation (Schumm et al., 2006; Lich et al., 2007; Zhang et al., 2007; Nadiminty et al., 2008). We have shown that treatment with both *Uncaria tomentosa* and LPS specifically activated p52. Treatment of THP-1 cells with *Uncaria tomentosa* and LPS also inhibited cell proliferation in a dose-dependent manner suggesting a linkage mediated through p52. Treatment of THP-1 cells with the NF-κB inhibitors AZT and SN50 did not completely block LPS-dependent cytokine expression and treatment with AZT did not alter LPS-dependent secretion of TNF-α and IL-1β.

Treatment with high doses of *Uncaria tomentosa* normally inhibited LPS-dependent TNF-α secretion by up to 80%. Similarly, treatment with the NF-κB inhibitor SN50 could also block TNF-α secretion by up to 80%. However, when the cells were treated with both the NF-κB inhibitor SN50 and *Uncaria tomentosa* the LPS-dependent TNF-α expression was increased by 40%. Treatment with SN50 significantly blunted the inhibition of TNF-α secretion by *Uncaria tomentosa* in LPS-treated cells. Cells treated with SN50, in the absence of LPS, did not affect TNF-α secretion. At lower doses of *Uncaria tomentosa,* treatment with SN50 did not alter LPS-dependent TNF-α secretion and TNF-α expression was inhibited by 75%. These results suggest that the activation of NF-κB subunits in response to *Uncaria tomentosa* treatment is mechanistically related to the suppression of TNF-α secretion.

Alternatively, treatment with the NF-κB inhibitor AZT monophosphate did not alter the level of secreted TNF-α or IL-1β (Fig. 5). AZT inhibits the phosphorylation of the NF-κB regulatory protein IκB and is a potent inhibitor of NF-κB nuclear transport and activation: 10 μg/ml AZT can inhibit NF-κB activity by 80–90% (Ghosh et al., 2003). Therefore, AZT inhibits classical NF-κB early in the pathway. These results suggest that *Uncaria tomentosa* affected NF-κB activation through a mechanism different from phosphorylation of the IκB regulatory molecule. This is supported by the observation that different subunits of NF-κB responded differentially to *Uncaria tomentosa*. Treatment of THP-1 cells with the NF-κB inhibitor, SN50, was able to block LPS-dependent secretion of TNF-α but did not affect LPS-dependent secretion of IL-1β. SN50 is a peptide that corresponds to the nuclear localization sequence of NF-κB subunits and competes for binding to the nuclear transport complex containing importin-α (Orange and May, 2008). This prevents p50 from being transported into the nucleus – because SN50 blocks the importin-α complex it can also inhibit the nuclear localization of other proteins including AP-1 and NFAT at high doses (Torgerson et al., 1998). These results suggest that the expression of TNF-α and IL-1β by THP-1 cells in response to LPS treatment is mediated by different NF-κB dimers. The current results also show that treatment with *Uncaria tomentosa* can differentially regulate TNF-α and IL-1β expression by mediating differential effects on NF-κB subunits.

In previous studies, we demonstrated that treatment with *Uncaria tomentosa* stimulated the expression of IL-1β both in the presence and absence of LPS. We have shown that treatment with *Uncaria tomentosa* enhanced IL-1β expression in a dose-dependent manner and that secretion of IL-1β was decreased by the inclusion of the NF-κB inhibitor SN50 ($p < 0.01$) but not by the inclusion of AZT. These results are further evidence that the mechanism of action of *Uncaria tomentosa* is related to the activity of specific NF-κB subunits.

The activation of NF-κB is also directly involved in the regulation of cellular apoptosis (Lu et al., 2009; Zhang et al., 2009). However, these data demonstrate that treatment of THP-1 cells with increasing doses of *Uncaria tomentosa* did not affect cell via-

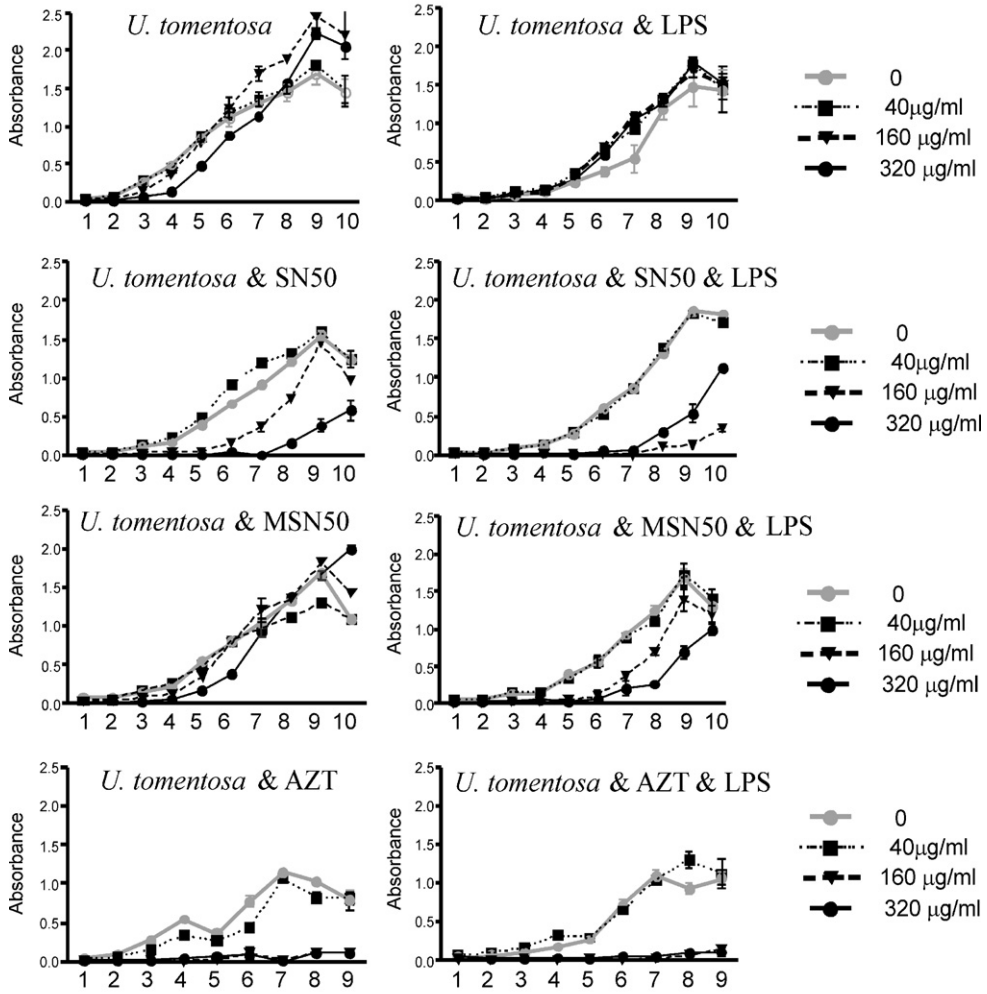

Fig. 6. Cell survival following treatment with various concentrations of *Uncaria tomentosa* and NF-κB inhibitors. THP-1 cells were treated with 0, 40, 160 or 320 μg/ml *Uncaria tomentosa* and 0 or 2.5 μg/ml LPS. Cells (10^3/well) were plated in quintuplicate onto 96-well plates. Every 24 h, cell viability was determined by the addition of 5×10^{-3} μg thiazolyl blue terazolium bromide per well. Following 4 h incubation, the purple formazide crystals were resuspended in 200 μl DMSO and the viability was determined as a measure of absorbance per sample as compared to untreated controls.

bility in spite of the ability of these levels of *Uncaria tomentosa* to affect NF-κB activity. Inhibition of NF-κB by treatment with the NF-κB inhibitors, SN50 or AZT, alone had relatively small effects on cell proliferation, although treatment with both *Uncaria tomentosa* and the NF-κB inhibitors resulted in a very significant decrease cell proliferation. In the presence of SN50, treatment with the highest dose of *Uncaria tomentosa* (320 μg/ml) significantly decreased cell viability ($p < 0.05$). In the presence of AZT, THP-1 cell proliferation was blocked at lower concentrations of *Uncaria tomentosa* (160 μg/ml). In addition, THP-1 cells treated with LPS also showed a small decrease in cell proliferation which, in the presence of the highest two doses of *Uncaria tomentosa*, was significantly lower than in untreated cells incubated with *Uncaria tomentosa* ($p < 0.01$). In cells treated with LPS, the ability of *Uncaria tomentosa* and the general NF-κB inhibitors to block cell proliferation was significantly enhanced. This suggests that in cells where NF-κB activation was blocked, *Uncaria tomentosa* dramatically enhanced cell death in immune-stimulated cells. Regulation of NF-κB can promote or prevent apoptosis depending on the cell type and activation status. *Uncaria tomentosa* inhibits all of the NF-κB subunits except p52, and prevents cell death in LPS-stimulated THP-1 cells (Figs. 3 and 6).

There are two activation pathways of NF-κB resulting in different cell processes, the classical pathway and the non-classical pathway. The classical NF-κB pathway is activated by LPS, TNF-α and IL-1β and leads to degradation of the inhibitory molecule IKBβ. This results in enhanced p65 and p50 activation which augments the inflammatory response through increased TNF-α expression, as well as promoting cell survival and tumourogenesis in malignant cells (Lich et al., 2007; Pickering et al., 2007). Alternatively, the non-classical pathway, which is less well characterized, is activated by CD40 and results almost exclusively in p52 processing following IKBα degradation. This leads to enhanced IL-1β expression, resulting in an increased inflammatory response (Solt et al., 2007). The expression of p52 in the non-classical NF-κB pathway results in increased cell survival but not in the promotion of metastasis (Solt et al., 2007; Wang et al., 2008).

Our findings suggest that *Uncaria tomentosa* mediates cellular regulation and cytokine expression through the p52 subunit of NF-κB, which is in support of recent publications that demonstrate that the expression and activation of p52 promotes cell survival and may differentially regulate the cellular response to anti-inflammatory or anti-cancer therapy (Schumm et al., 2006; Nadiminty et al., 2008).

The activation of immune cells, such as monocyte/macrophages, generally involves the activation of pro-inflammatory transcription factors, primarily NF-κB and AP-1 (Zhang and Ghosh, 2000; Granet et al., 2004). Once activated, inflammatory factors including TNF-α and IL-1β are produced, which further stimulates the inflammatory response. This chronic inflammatory process is involved in a variety of disorders including rheumatoid arthritis, atherosclerosis, and tumour formation. There is a complex interaction between immune activation and tumour growth. The infiltration of chronically activated immune cells may not target the tumor for destruction but rather activate proliferation by providing necessary vascularization and growth factors required for tumor formation (Hagemann et al., 2005). Since the NF-κB pathway leading to activation of TNF-α is critical not only for the inflammatory response but also for tumor metastasis, this pathway provides an excellent target for novel drug development and therapeutic intervention. *Uncaria tomentosa* is a promising candidate for further studies in this area.

Uncaria tomentosa is an effective anti-inflammatory that decreases the immune dependent expression of TNF-α, and activation of both AP-1 and the classical NF-κB pathway. The mechanism of action of *Uncaria tomentosa* appears to be largely regulated though the activation of the p52 subunit of NF-κB, as shown by the subunit activation and inhibitor studies, as well as by the enhanced IL-1β and inhibited TNF-α expression. We also confirm previous findings that demonstrated that inhibition of the classical NF-κB pathway, which results in decreased TNF-α expression, can block cell proliferation by high doses of *Uncaria tomentosa*. This highlights the potential therapeutic uses for *Uncaria tomentosa* in anti-cancer regimes, and anti-inflammatory properties.

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