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Inhibition of TLR Ligand- and Interferon Gamma-Induced Murine Microglial Activation by *Panax Notoginseng*

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Abstract

Among the many products which influence microglial activation and resulting neuroinflammation, herbal medicine has recently drawn much attention due to its immunomodulatory and neuroprotective activities. The purpose of the current study was to investigate the effects of an extract of *Panax notoginseng* (NotoG™) on TLR ligand-and IFN γ -induced activation in N9 and EOC20 microglial cells lines. NotoG suppressed microglial activation as measured by reduced expression of accessory molecules (CD40 and CD86), decreased production of inflammatory mediators (IL-6 and TNF α), and diminished release of antibacterial products (nitric oxide). Furthermore, this immunosuppressive activity was neither dependent on the glucocorticoid receptor, nor the result of a single ginsenosides (Rb1, Rg1, or Re), which are the major active constituents of the whole extract. NotoG and select ginsenosides may therefore be of therapeutic benefit in treating or preventing neurodegenerative diseases such as multiple sclerosis and parkinson's disease.

Keywords

Inflammation; Cytokine; Nitric oxide; Glucocorticoid receptor; Ginsenoside

Introduction

Microglia (M ϕ), the resident antigen presenting cell within the central nervous system (CNS), play a significant role in host defense and tissue repair within the CNS. As the CNS' intrinsic immune effector cell, M ϕ rapidly respond to subtle, acute, and chronic pathological stimuli. However, recent evidence has established that M ϕ also contribute to neuro-pathological changes associated with several CNS diseases including Alzheimer's Disease (AD), Parkinson's Disease (PD), Multiple Sclerosis (MS), Acquired Immune Deficiency Syndrome Dementia (HIV-Dementia), Traumatic Brain Injury (TBI), and Hypoxic-Ischemic Injury (Dheen et al. 2007; Esiri 2007; Glezer et al. 2007; Peterson and Fujinami 2007).

Although once considered separate and distinct events in the same disease, inflammation and neurodegeneration are now known to occur in concert within the same CNS pathology (Esiri 2007).

As the primary immune effector cell in the CNS, MÖ migrate to the site of tissue injury where they respond to invading pathogens or other inflammatory signals. Like peripheral monocytes and macrophages, MÖ become activated and participate in propagation of CNS inflammation through antigen presentation and the production of pro-inflammatory and toxic mediators (Suk 2005; Ponomarev et al. 2006). In vitro, the process of MÖ activation is associated with upregulation of cell surface markers such as CD45, MHC class II, CD40, and CD86 (Aloisi et al. 2000; Ponomarev et al. 2005); although this has not been extensively characterized in vivo. Activation of MÖ and the consequent release of inflammatory mediators such as interleukin 1 (IL-1), tumor necrosis factor alpha (TNF- α), glutamate, nitric oxide (NO), and reactive oxygen species (ROS) may contribute to neurodegenerative processes (Aloisi et al. 2001; Glezer et al. 2007). In vitro studies demonstrated MÖ activation increased neurotoxicity through the production of pro-inflammatory and cytotoxic mediators in mixed neuron-glial cultures stimulated with either toll like receptor (TLR) ligands (e.g. lipopolysaccharide, LPS), cytokines (e.g. TNF- α or IFN- γ) glutamate, N-methyl-D-aspartate (NMDA), or β -amyloid (A β) (Dheen et al. 2007; Glezer et al. 2007). Activation of MÖ in the CNS may initially be intended to protect neurons; however, more frequently, activation of these cells and inflammatory products derived from them have been implicated in various disease states (Suk 2005). Therefore, activation of MÖ occupies a key position whereby the immune response may proceed in two directions: one being neuroprotective and the other neurotoxic (or a combination of the two), depending on the stimulus. In this manner, suppression of MÖ activation represents an important immunotherapeutic target.

Among the many endogenous and exogenous factors that might influence MÖ activation and resulting neuroinflammation, herbal medicine has recently drawn much attention due to its potential inhibitory effects on inflammatory responses and neuroprotective activity (Radad et al. 2006; Rausch et al. 2006; Rhule et al. 2006). Because of its wide range of pharmacological effects, *Panax ginseng* has been used in traditional Chinese medicine for more than 5 thousand years as both a tonic and a haemostatic agent (Radad et al. 2006). As such, it continues to occupy a prominent position on the herbal best seller list and is considered the most widely consumed herbal product in the world (Blumenthal 2001; Barnes et al. 2004). Ginseng saponins, also known as ginsenosides, are the principal active components responsible for ginseng's biological effects. The biological activity of the species *Panax Notoginseng* is similar to the more widely known *Panax ginseng*, with differences in activity associated with higher levels of ginsenosides in the *Panax Notoginseng* species (Zhu et al. 2004). Pharmacological effects of ginseng and ginsenosides have been demonstrated on the CNS, as well as the cardiovascular, endocrine and immune systems (Attele et al. 1999; Radad et al. 2006; Lopez et al. 2007); however, the ability of ginsenosides to act on CNS immune cells has not been widely examined.

CNS effects include increased cell survival, extension of neurite growth, and rescuing of neurons from death in consequence of different insults both in vivo and in vitro (Radad et al. 2006; Rausch et al. 2006). A number of studies have begun to describe the beneficial effect of ginseng and ginsenosides in neurodegenerative disease models (e.g. Parkinson's disease, spinal cord injury, and Ischemia-reperfusion injury) which are thought to occur via inhibition of inflammation and MÖ activation in particular (Bu et al. 2005; Lin et al. 2007; Joo et al. 2008). Although the ability of ginseng and ginsenosides to modulate some inflammatory and allergic processes has been documented within the peripheral immune system, to date there is a shortage of studies examining ginseng's ability to modulate

neuroinflammatory conditions (Lin et al. 2007; Wu et al. 2007; Joo et al. 2008). We hypothesized that *Panax notoginseng* may temper MÖ activation processes. In this study, we examined whether an extract of *Panax Notoginseng* (NotoG™) attenuates TLR ligand- or interferon gamma (IFN γ)-induced activation of MÖ. In vitro activation of murine MÖ by both TLR ligands and IFN γ was suppressed by concomitant NotoG treatment in a dose-dependent and ligand specific manner with regards to cell surface molecules and secreted cytokines. Furthermore, this immunosuppressive activity was neither dependent on the glucocorticoid receptor, nor the results of a single ginsenoside component of the whole extract.

Materials & methods

Plant extracts

Extracts from the plant *Panax Notoginseng* (Noto-G™) were kindly provided by Technical Sourcing International, Inc. (TSI, Missoula MT). Notoginseng was extracted from the root of the *Panax notoginseng* plant using ethanol and standardized to contain Rb1 and Rg1 ginsenosides at 35% and 34% of the whole extract, respectively. Quantification of Rb1 and Rg1 content were determined by high performance liquid chromatography analysis (HPLC) by TSI and NotoG extracts were determined to be free of *E. coli* and *S. enterica* contamination (data not shown). The lyophilized NotoG extract, purified ginsenosides Rb1 and Rg1 (Rg1 34 μ g/ml, Rb1 32 μ g/ml, MP Biomedicals Solon, OH), purified Re (9 μ g/ml Sigma St Louis, MO), Lipopolysaccharide (LPS) from *E. coli* (O55:B5, 100 ng/ml, Sigma), CpG ODN 1826 type B specific for mouse TLR9 (0.5 μ M, Invivogen San Diego, CA), Poly I:C (a synthetic analog of dsRNA, 250 μ g/ml Invivogen), IFN γ (5 ng/ml, RnD Systems Minneapolis, MN), RU486 (5 mM Sigma), and dexamethasone (6.8 mM, Sigma) were dissolved in complete RPMI (see below).

Cell culture and microglial stimulation

The murine N9 MÖ cell line, originally derived from CBA mice, was kindly provided by Dr. P Ricciardi-Castagnoli (University of Milano-Bicocca, Milan Italy). The cells were maintained in complete RPMI 1640 medium supplemented with 3% heat inactivated FBS, 2 mM glutamine, 25 mM HEPES, with antibiotic-antimycotic solution (Sigma). The murine EOC20 MÖ cell line, originally derived from C3H/HeJ juvenile mice, was purchased from ATCC (CRL-2469). The cells were maintained in DMEM with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10%; heat inactivated FBS, 20% LADMAC Conditioned Media (produced from the LADMAC cell line (CRL-2420, ATCC), with antibiotic-antimycotic solution (Sigma). MÖ were seeded at 10^6 cells/ml/well of a six well plate and immediately exposed to NotoG (1-100 μ g/ml, TSI), LPS (endotoxin, 100 ng/ml), CpG (0.5 μ M), Poly I:C (250 μ g/ml), IFN γ (5 ng/ml), RU486 (5 mM), dexamethasone (6.8 mM), Re (9 μ g/ml), Rg1 (34 μ g/ml), and Rb1 (32 μ g/ml) and allowed to incubate for 24 h at 37°C. To minimize variability MÖ cells were lightly scraped within the spent culture media, collected into 1.5 ml eppendorf tubes, centrifuged, and the media and cells separated for further analysis.

Flow cytometry

MÖ were harvested, washed, and non-specific antibody binding blocked with purified rat anti-mouse CD16/CD32 (BD Pharmingen, San Jose, CA) diluted 1:100 in 30 μ g of rat IgG (Jackson ImmunoResearch, West Grove, PA) prior to staining with fluorochrome-conjugated antibodies. One μ g of monoclonal antibodies specific to CD40 PE (clone 3/23), CD86 APC clone GL1), BD Pharmingen), CD54 Pacific Blue (clone YN1/1.7.4, Biolegend, San Diego, CA), MHC class II FITC (clone M5/114.15.2), CD38 PE-Cy7 (clone 90), CCR7 PE (clone 4B12), or CCR3 APC (clone 5E8-G9-B4, eBioscience, San Diego, CA) were

added and incubated for 30 min on ice. Five μ l of 7-AAD (BD Pharmingen) was then added directly to the cells and incubated for 5 min at room temperature. Cells were washed with PBS, re-suspended in 0.3 ml PAB (1% bovine serum albumin, 0.01% sodium azide in PBS) and analyzed immediately. Cell acquisition and analysis was performed on a FACS Aria flow cytometer using FACS Diva software (version 4.1.2). A live cell gate was established using 7-AAD negative cells and 100,000 events captured. Compensation of the spectral overlap for each fluorochrome was calculated using anti-rat/hamster IgG compensation beads (BD Biosciences).

Cytokine ELISA, LDH, and nitrite assay

Acellular supernatants resulting from the experiments described above were collected and analyzed for IL-6 and TNF α production using murine cytokine ELISA kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). In addition, 50 μ l of tissue culture supernatants were analyzed for LDH (Biovision, Mountain View, CA) or nitrite levels (NO $_2^-$) according to the Griess method following the manufacturer's instructions (Promega, Madison, WI).

Data analysis

For each parameter, the values for individual experiments were averaged and the standard deviation and standard error calculated. The significance of the differences between the exposure groups was determined by *t*-test, one-way, or two-way ANOVA, in conjunction with Bonferroni's post-hoc analysis and Tukey's test for variance, where appropriate. All ANOVA models were performed with Prism software, version 4. A *P* value of <0.05 was considered significant.

Results

Notoginseng inhibits LPS-induced activation of N9 microglia in a dose-dependent manner

After microglia (M ϕ) encounter pathogens, toll like receptor (TLR) mediated activation results in increased cell surface expression of accessory molecules, as well as increased production of pro-inflammatory cytokines and anti-bactericidal activities which are essential to initiating and maintaining a proper immune response (Olson and Miller 2004). To test whether concurrent exposure of N9 M ϕ to endotoxin (LPS, the classical TLR4 ligand) and NotoG resulted in altered expression of accessory molecules and early response cytokines in a dose-dependent manner, cells were stimulated with LPS and varying concentrations of NotoG for 24 h. As anticipated, LPS increased the expression of CD40 on N9 M ϕ when compared to unstimulated cells as measured by flow cytometry (Fig. 1a). LPS-induced upregulation of CD40 expression was attenuated by co-exposure with 25, 50, and 100 μ g/ml, but not 5 μ g/ml NotoG (Fig. 1a). While LPS stimulation slightly reduced CD86 expression compared to unstimulated M ϕ , NotoG increase CD86 expression at all concentrations examined compared to LPS alone (Fig. 1b). Cytokine ELISAs established that N9 M ϕ increased production of IL-6 and TNF α in response to LPS stimulation (Fig. 1c and d, respectively). This increase in pro-inflammatory cytokine production was diminished by concomitant treatment with 50 and 100 μ g/ml NotoG. Although 5 and 25 μ g/ml NotoG exhibited a trend towards a reduction in IL-6 and TNF α , this decrease was not statistically significant (Fig. 1c and d, respectively). With regards to accessory molecule expression, only 100 μ g/ml NotoG alone altered expression of CD40 and CD86 compared to unstimulated M ϕ . No differences were observed in either IL-6 or TNF α production in response to any of the concentrations of NotoG alone. Furthermore, because the MFI analysis was performed on live cells (gated as 7-AAD negative) (data not shown) and because no variation was detected in either percent cell viability, LDH release, or the Aqueous One cell viability assay (data not shown), the differences detected in LPS-induced

activation by NotoG treatment were not attributed to cytotoxicity. Although 100 µg/ml of NotoG was the most potent of the concentrations examined, 50 µg/ml NotoG was chosen for analysis in the subsequent studies because of its ability to reduce MÖ activation, without any inherent effects of its own.

Notoginseng attenuates TLR ligand and IFN γ -induced nitric oxide production

Nitric oxide (NO) is a major mediator of innate immune responses due to its involvement in bactericidal activity (Delclaux and Azoulay 2003), plays a role in the regulation of cytokine synthesis (Coleman 2002), and promotes neuro-degeneration (Xie et al. 2002). In order to investigate the impact of NotoG on NO production in response to the TLR ligands: LPS, CpG, Poly I:C, and the potent microglial activator interferon gamma (IFN γ), the levels of nitrite (NO $_2^-$), which is one of two primary, stable and nonvolatile breakdown products of NO, were measured. Only trace amounts of NO $_2^-$ were detected in the acellular supernatants collected from unstimulated MÖ. These values were not altered by NotoG alone in either N9 or EOC20 MÖ (data not shown). Exposure to LPS, CpG, and IFN γ , but not Poly I:C, increased NO $_2^-$ levels compared to unstimulated N9 MÖ. Concomitant exposure to NotoG attenuated LPS-(42.1%), CpG- (21.7%), and IFN γ -induced NO $_2^-$ production (18.8%) compared to the stimulant alone (Fig. 2a). In contrast, CpG, Poly I:C, and IFN γ , but not LPS, increased NO $_2^-$ compared to unstimulated EOC20 MÖ. Similarly, simultaneous exposure to NotoG decreased CpG- (30.1%), Poly I:C- (26.6%), and IFN γ -induced NO $_2^-$ production (10.1%) compared to the stimulants alone (Fig. 2b).

CD40 accessory molecule expression is attenuated by notoginseng

Stimulation of MÖ upregulates the expression of cell surface antigens involved in T cell activation. Of these, the CD40 molecule in particular, has an important role in promoting inflammatory responses by macrophages/microglia, since interaction with its cognate ligand, CD154, leads to secretion of cytokines and neurotoxins which contributes to neuro-immunologic pathologies in the CNS. To investigate whether NotoG modulates the expression of CD40 on mu-rine MÖ cell lines, the levels of CD40 were analyzed by flow cytometry on N9 and EOC20 MÖ following co-culture with LPS, CpG, Poly I:C, or IFN γ . NotoG alone slightly augmented CD40 expression on N9 MÖ (Fig. 3a), but had no effect on EOC20 MÖ (Fig. 3b). As anticipated, LPS and IFN γ increased expression of CD40 on N9 MÖ; whereas concomitant exposure to NotoG decreased CD40 expression by 25.0% and 47.5%, respectively (Fig. 3a). In contrast, in the presence of CpG and Poly I:C the expression of CD40 accessory molecules was similar to the unstimulated N9 MÖ, but augmented by the presence of NotoG relative to the stimulant alone (16.8 and 23.1%, respectively) (Fig. 3a). EOC20 MÖ were responsive to CpG-, Poly I:C- and IFN γ -induced activation, but not LPS, and consequently upregulated expression of CD40 (Fig. 3b). Similar to the observed response in N9 MÖ, in the presence of NotoG, EOC20 MÖ simultaneously stimulated with CpG- (11.4%), Poly I:C-(20.7%), or IFN γ 36.7 showed reduced expression of CD40 compared to the stimulant alone (Fig. 3b).

Pro-inflammatory cytokine production is reduced by notoginseng

Both TLR ligands and IFN γ stimulate the production of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF α). To determine whether NotoG modifies the production of pro-inflammatory cytokines by MÖ, the amount of IL-6 and TNF α produced by N9 and EOC20 MÖ cell lines was determined following co-culture with LPS, CpG, Poly I:C, or IFN γ . As anticipated, Fig. 4 demonstrates that pro-inflammatory cytokine production is upregulated in response to TLR ligands and IFN γ in both MÖ cell lines. N9 MÖ increased production of IL-6 and TNF α in response to LPS, CpG, and IFN γ , but not Poly I:C (Fig. 4a & b). Consistent with the observed effects on NO $_2^-$ and accessory molecule expression, concomitant exposure to LPS, CpG, or IFN γ and NotoG attenuated

IL-6 and TNF α cytokine production compared to the stimulant alone (Fig. 4a & b). EOC20 M ϕ upregulate IL-6 production in response to LPS, CpG, and Poly I:C and this increase is reduced by concomitant exposure to NotoG (Fig. 4c & d). In contrast, TNF α production by EOC20 M ϕ in response to CpG and Poly I:C stimulation exhibits augmented levels of TNF α in the presence of NotoG (Fig. 4c & d). The percent change from control values are indicated above the corresponding bars on the graph.

Chemokine receptor expression induced by TLR ligand is not affected by notoginseng

Activated M ϕ phagocytose damaged cells and debris, as well as foreign pathogens, and subsequently express surface proteins involved in maturation and migration including chemokine receptors such as CCR7 (Aloisi 2001; Dijkstra et al. 2006). Table 1 demonstrated that chemokine receptors are upregulated in response to LPS on N9 M ϕ , but not in response to Poly I:C on EOC20 M ϕ . These data show that NotoG alone slightly increased CCR7 and CD38 expression on the cell surface of N9 M ϕ , while no effect was observed with CCR3. NotoG augmented CD38 expression in the presence of LPS, but had no effect on either CCR7 or CCR3 expression (Table 1). Interestingly, EOC20 M ϕ responded to Poly I:C challenge by reducing expression of CCR7 and CCR3, while no effect was observed on CD38 levels. Of the three molecules examined on EOC20 M ϕ , only CCR3 expression was affected by NotoG treatment.

NotoG does not appear to act through the glucocorticoid receptor

Ginseng and ginsenoside extracts exhibit glucocorticoid-like activities in homeostasis and regulation of immunity (Chung et al. 1998; Ling et al. 2005). Because the actions of glucocorticoids are mediated through cytosolic glucocorticoid receptors (GRs), we hypothesized that NotoG may mediate its immunosuppressive actions by binding to the GR. Concomitant exposure of N9 M ϕ cells to LPS or IFN γ and the glucocorticoid receptor antagonist RU486 attenuated CD40 expression compared to the stimulant alone (Fig. 5a and b). Furthermore, the immunosuppressive effects of NotoG against LPS-induced activation were not blocked by RU486, indicating that the action of the whole NotoG extract is not mediated through GR (Fig. 5a). In contrast, the ability of NotoG to reduce IFN γ -induced CD40 expression was not reversed by RU486, but rather synergistically suppressed by RU486 (Fig. 5b). In the LPS stimulated cells, dexamethasone was a more potent inhibitor of CD40 expression than NotoG (Fig. 5a); while in IFN γ stimulated cells NotoG appeared to be the more potent inhibitor (Fig. 5b). NotoG also appeared to have a greater ability to suppress CD40 expression in response to IFN γ stimulation than dexamethasone (Fig. 5b).

Re, Rb1, and Rg1 ginsenosides exert differential effects LPS- and IFN γ -induced M ϕ activation

Ginsenosides, which are triterpene saponins that have a rigid steroidal skeleton with sugar moieties, are purported to be the pharmacologically active component of ginseng (Leung et al. 2006; Leung et al. 2007a, b). In order to determine the contributions of the three major ginsenosides present in NotoG to the observed effects of the whole extract on CD40 expression, the response of N9 M ϕ cells exposed to LPS or IFN γ in the presence or absence of Rg1, Rb1 or Re ginsenosides was examined. Purified ginsenosides at comparable concentrations to those found in the whole extract exhibited no effects on CD40 expression (Fig. 6); however, differential effects on M ϕ activation were observed dependent on the stimulant. LPS-induced elevations in CD40 expression were attenuated by the ginsenoside Rg1, but not Rb1 or Re (Fig. 6a). In contrast, IFN γ -induced elevations in CD40 expression were attenuated by the ginsenoside Re, but not Rb1 or Rg1 (Fig. 6b). Although none of the major components of our NotoG extract individually attenuated IFN γ -induced nitrite production by N9 M ϕ , each of the three ginsenosides (Re, Rb1, and Rg1) may contribute to

the observed ability of the whole NotoG extract to reduce IFN γ -induced nitrite production (Table 2).

Discussion

Activation of MÖ and the consequent release of inflammatory mediators such as interleukin 1 (IL-1), tumor necrosis factor alpha (TNF- α), glutamate, nitric oxide (NO), and reactive oxygen species (ROS) contribute to neurodegenerative processes (Aloisi et al. 2001; Glezer et al. 2007) as demonstrated in pathological lesions from Alzheimer's Disease (AD), Parkinson's Disease (PD), Multiple Sclerosis (MS), Acquired Immune Deficiency Syndrome Dementia (HIV-Dementia), Traumatic Brain Injury (TBI), and Hypoxic-Ischemic Injury (Dheen et al. 2007; Glezer et al. 2007; Peterson and Fujinami 2007). Although pharmacological effects of *Panax ginseng* and ginsenosides have been demonstrated on the CNS, as well as the cardiovascular, endocrine and immune systems (Attele et al. 1999; Radad et al. 2006), the ability of *Panax notoginseng* to act on CNS immune cells such as MÖ has not been widely examined (Lopez et al. 2007; Wu et al. 2007). Therefore, the present studies were designed to determine whether MÖ activation stimulated via innate immune receptors (e.g. TLR) and interferon gamma (IFN γ) was modulated following exposure to an ethanolic extract of *Panax notoginseng* (NotoG). NotoG reduced both TLR ligand- and IFN γ -induced activation of MÖ in a dose-dependent manner by reducing cell surface expression of accessory molecules, inflammatory cytokines, and release of bactericidal products (e.g. nitric oxide). The findings of the present study also demonstrate that NotoG reduced TLR ligand- and IFN γ -induced activation was not mediated via glucocorticoid receptors. Furthermore, the contributions of specific ginsenosides (e.g. Re, Rb1, and Rg1) to these inhibitory effects of NotoG on CD40 expression may be dependent on the stimulant utilized.

There exists an urgent need for the generation and development of novel therapies and/or adjuvants to current treatment available for numerous neurodegenerative and neuroimmune diseases. Although a variety of compounds have been used in experimental therapies for these conditions, most of these materials elicit an immune response in the periphery only, exhibit limited ability to cross the blood brain barrier, and/or have undesirable side effects that have limited their potential application. Natural products are currently receiving widespread recognition as having beneficial health effects when consumed on a regular basis as part of a varied diet and at effective levels (Suk 2005). Mounting evidence from epidemiological studies, animal research, clinical trials and research in nutritional biochemistry suggests that natural products may be beneficial in coronary heart disease, cancer, osteoporosis, diabetes, Parkinson's and Alzheimer's diseases. These studies indicate that the mechanistic actions of natural products involve a wide array of biological processes, including activation of antioxidant defenses, signal transduction pathways, cell survival-associated gene expression, cell proliferation and differentiation, and preservation of mitochondrial integrity. Furthermore, many natural products exert anti-inflammatory actions through inhibition of oxidative stress-induced transcription factors (e.g., NF- κ B, AP-1), cytotoxic cytokines and cyclooxygenase-2 (Mandel et al. 2005). Therefore, natural products and/or their component compounds that target specific pathways involved in MÖ activation may halt or limit the progression of neuroinflammatory and neurodegenerative disorders.

Pharmacological effects of ginseng and ginsenosides have been demonstrated on the cardiovascular, endocrine and immune systems (Attele et al. 1999; Radad et al. 2006). In particular, *Panax Notoginseng* (commonly referred to as Notoginseng) has been shown to exhibit a wide range of actions in the CNS including increased cell survival, extension of neurite growth, and rescuing of neurons from death following both in vivo and in vitro insults (Jiang and Qian 1995; Chen et al. 2003; Radad et al. 2006; Lopez et al. 2007).

Although the ability of Notoginseng to act on immune cells within the CNS has not been widely examined, collectively, scientific evidence supports the hypothesis that microglial-neuronal interactions are critically involved in neuroinflammatory and neurodegenerative disorders, thereby providing a target for therapeutic intervention by Notoginseng (Rock and Peterson 2006). Previous studies and the data presented herein suggest that an ethanolic extract of *Panax Notoginseng*, NotoG reduces 1) the expression of immune molecules expressed on the cell surface, 2) the production of inflammatory cytokines and nitric oxide production, and 3) modulates signal transduction pathways necessary to mediate innate immune responses by multiple APC types (Rhule et al. 2006). Therefore, *Panax Notoginseng* represents a novel natural alternative and/or complementary approach to current traditional medicines to reduce the detrimental effects of MÖ activation.

The innate immune response within the CNS is directed at identifying pathogens as non-self by recognizing pathogen associated molecular patterns through various toll like receptors (TLR) (Lee and Kim 2007). The broad expression pattern of mRNA for nine identified TLR in cultured neonatal MÖ and the functional activation of these cells following stimulation with TLR agonists further implicates MÖ as playing an important role in the CNS innate immune system (Olsson et al. 2006). TLR3 recognizes double stranded RNA found primarily in viruses, TLR4 recognizes LPS in association with LPS-binding protein, CD14 and MD-2, and TLR9 recognizes bacterial CpG DNA (Amati et al. 2006). TLR are expressed on a variety of cells; however, most cells have preferential expression of TLR. The results from our study suggest that N9 and EOC20 MÖ differ in their TLR expression based on differential sensitivity to three TLR ligands. This observation may be directly related to the original strain of mice from which the cells were derived. Because of a point mutation at a single residue in the cytoplasmic tail of TLR4, signaling via TLR4 in C3H/HeJ macrophages is deficient; therefore, the murine EOC20 MÖ cell line (originally derived from C3H/HeJ juvenile mice) would not be expected to respond to LPS (Poltorak et al. 1998). Although in the current study, minimal activation of N9 and EOC20 MÖ by CpG was observed, several studies previously demonstrated that primary MÖ, as well as BV-2 and N9 MÖ cell lines express TLR9 and can be activated by CpG-ODN to express different cytokines (Dalpke et al. 2002; Zhang et al. 2005). It would appear that in our hands, EOC20 MÖ preferentially express TLR3 over TLR9, as exhibited by the more robust response to Poly I:C than CpG. Although we have not yet directly measured and compared the expression levels of TLR3, TLR4, and TLR9 in N9 vs. EOC20 MÖ cells lines, studies are currently underway to examine TLR expression levels and determine whether these levels correlate with the biological responses observed.

TLR ligands including LPS, CpG, and Poly I:C, as well as IFN γ , are potent inducers of accessory molecules such as MHC class II, CD40, CD54 and CD86 on immune cells including MÖ. These accessory molecules then function to facilitate the recruitment, interaction, and activation of lymphocytes. When stimulated with TLR ligands or IFN γ , N9 and EOC20 MÖ cell lines respond by upregulating the cell surface expression of these accessory molecules. The current study demonstrates that an ethanolic extract of *Panax Notoginseng* (NotoG) decreased LPS-induced CD40 accessory molecule expression by N9 MÖ in a dose-dependent manner; yet also appeared to act synergistically with LPS to increase CD86 expression. Subsequent more detailed studies showed that NotoG diminished TLR ligand- and IFN γ -induced CD40 by N9 and EOC20 MÖ. This apparent dichotomy suggests that the NotoG extract may act on multiple, distinct pathways or that different ginsenoside components of the whole NotoG extract may act in opposing manners—which is consistent with other natural products, as well as the constituents of the current NotoG extract (Sengupta et al. 2004; Joo et al. 2005; Mandel et al. 2005). Although previous studies demonstrated that TLR4 and CD14 expression was unaffected by NotoG (Rhule et al. 2006),

we have not yet assessed the expression of TLR3 or TLR9 in response to NotoG in murine microglial cell lines.

Several of the secretory products that play a direct or indirect anti-microbial role have been implicated in the pathogenesis of neuroinflammatory and neurodegenerative disorders, including reactive oxygen and reactive nitrogen species (Schell et al. 2007; Wu et al. 2007). Of these, nitric oxide is a key messenger involved in physiological functions, as well as regulation of inflammatory and immune responses (Willenborg et al. 2007). To characterize the ability of NotoG to alter the MÖ antibacterial response to exogenous TLR ligand and IFN stimulation, we performed assays to examine nitrite production. NotoG alone exhibited no stimulatory effect on nitrite levels—similar to the effects of purified ginsenosides on LPS induced NO production (Wu et al. 2007). Our results showed that TLR ligand- and IFN -dependent increases in nitrite production were reduced by concomitant exposure to NotoG in both cell lines examined. Previous studies using N9 MÖ showed that the ginsenosides Rg1 and Re1, but not Rb2 or Rd, decreased LPS-induced nitrite levels, but did not examine effects on CpG, Poly I:C, or IFN induction (Wu et al. 2007). Although neither of the major components of our NotoG extract (Rb1, Rg1, and Re) individually attenuated IFN -induced nitrite production by N9 MÖ, we have not yet studied the effects of these ginsenosides alone on TLR ligand-induced nitrite production. It appears that each of the three ginsenosides contribute in small fashion to the ability of the whole NotoG extract to reduce IFN -induced nitrite production. Taken together, these data suggest that NotoG may be neuroprotective against various CNS pathologies by limiting the activation of MÖ by microbial pathogens.

Although the exact function of MÖ is not fully understood, several lines of evidence suggest that these cells may sustain and propagate inflammation within the CNS through antigen presentation and/or cytokine/chemokine secretion (Ponomarev et al. 2005; Ponomarev et al. 2006; Muzio et al. 2007). During disease pathology, activated MÖ secrete potent pro-inflammatory cytokines such as TNF, IL-6 and IL-1 and a recent study demonstrated the ability of specific ginsenosides to reduce LPS-induced TNFa secretion (Wu et al. 2007). We expanded upon these observations to show that NotoG reduces IL-6 and TNFa secretion in response to three different TLR ligands, as well as IFN in two distinct MÖ cell lines. Given that transcription factors such as NF-kB are critical regulators of inflammatory genes in MÖ (Bhat et al. 1998; Chan et al. 2001), and that the ginsenoside Rg3 reduced A₄₂-mediated microglial activation through inhibition of NF-kB p65 binding (Joo et al. 2008), the capacity of NotoG to affect these systems will be examined in further investigations into its molecular mechanisms of action.

Because ginseng and ginsenosides have previously been shown to exhibit glucocorticoid-like activities in homeostasis and regulation of immunity (Chung et al. 1998; Ling et al. 2005) and the biological actions of glucocorticoids are mediated through cytosolic glucocorticoid receptors (GRs), we examined the ability of NotoG to exert its immunosuppressive effects through these receptors. Using the glucocorticoid receptor antagonist RU486, our studies indicated that the action of the whole NotoG extract is not likely mediated through GR. Although the combination exposure of LPS or IFN plus or minus NotoG in the presence of absence of RU486 yielded contrasting results, this most likely may be attributed to the nature of the compound itself—which has both partial agonist and antagonist properties.

Ginsenosides, which have a rigid steroidal skeleton with sugar moieties—are purported to be the pharmacologically active component of ginseng (Leung et al. 2006; Leung et al. 2007a; Leung et al. 2007b). Purified ginsenosides at comparable concentrations to those found in the whole extract exhibited no effects on CD40 expression; however, differential effects on

MÖ activation were observed dependent on the stimulant. Ginsenoside Rg1 appeared to attenuate LPS-induced elevations in CD40 expression, while ginsenoside Re reduced IFN -induced elevations in CD40 expression. Although neither Re, Rb1 or Rg1 individually reduced IFN -induced nitrite production by N9 MÖ, each of the three ginsenosides (Re, Rb1, and Rg1) may contribute to the observed ability of the whole NotoG extract to reduce IFN -induced nitrite production.

An important aspect of our study is the use of MÖ cell lines, as opposed to primary murine MÖ. While cell lines are an invaluable tool for defining key steps in activation responses, evaluating otherwise unmanipulated, pure primary cultures represents a more powerful tool to resolve the basis of cellular activation in disease pathologies and immunosuppression in response to natural products such as NotoG. Thus, as our studies and understanding progress, the use of primary MÖ will be essential, not only to reveal how altered signaling impacts the eventual CNS immune response, but also to ascertain whether potential differences exist in distinct MÖ populations. Moreover, there exists a need to evaluate the use of Notoginseng and its components in various animal models of disease (Suk 2005).

Collectively, these studies confirm a critical role for MÖ in the innate immune response to CNS pathogens, and more importantly suggests that an ethanolic extract of *Panax Notoginseng* (NotoG) attenuated CNS immune functions in response to TLR ligands as a model of pathogen exposure. MÖ have been suggested to play a role in various CNS diseases such as Multiple Sclerosis, Alzheimer's disease, bacterial meningitis, and ischemia-reperfusion injury. In particular, MS has been suggested to be initiated or exacerbated by the presence of underlying infections (Becher et al. 2001). It may be that Notoginseng's ability to reduce TLR ligand- and IFN -induced MÖ activation represents a novel therapeutic target for the modulation of immune functions in MS and other CNS inflammatory or neurodegenerative diseases. Therefore, a better understanding of the mechanism (s) by which Notoginseng modulates MÖ activation using in vitro and in vivo tools may lead to the development of additional therapeutics strategies aimed at attenuating immune activation.

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Fig. 1. Notoginseng (NotoG) decreased LPS-induced activation of N9 MÖ in a dose-dependent manner. LPS increased expression of CD40 (**a**), but not CD86 (**b**) compared to unstimulated MÖ. This increased CD40 expression was dose-dependently reduced by NotoG, and 100 μ g/ml of NotoG was the most potent of the concentrations examined. In contrast, LPS and NotoG appeared to act synergistically to increase CD86 expression. As anticipated, LPS activation increased the production of IL-6 (**c**) and TNF (**d**) by N9 MÖ. Similarly, this upregulation of pro-inflammatory cytokines was attenuated by NotoG. $n=3-6$; in triplicate. Error bars indicate the standard error of the mean. * $P<0.05$ compared to unstimulated control (dashed line). # $P<0.05$ compared to LPS only control

Fig. 2.

Notoginseng reduced TLR ligand and IFN γ -induced nitrite production by both N9 and EOC20 MÖ. NotoG alone exhibited no stimulatory effect on nitrite levels. **a** LPS, CpG, and IFN γ , but not Poly I:C, increased nitrite production compared to unstimulated N9 MÖ. This increase was reduced by concomitant exposure to 50 μ g/ml NotoG. **b** CpG, Poly I:C, and IFN γ , but not LPS, increased nitrite production compared to unstimulated EOC20 MÖ. This increase was similarly reduced by concomitant exposure to NotoG. $n=3-6$; in triplicate. Error bars indicate the standard error of the mean. * $P<0.05$ compared to unstimulated control (dashed line). # $P<0.05$ compared to appropriate TLR ligand control

Fig. 3.

Notoginseng attenuated TLR ligand and IFN γ -induced CD40 expression by MÖ. NotoG alone exhibited slight stimulatory effects on CD40 levels. **a** LPS and IFN γ , but not CpG or Poly I:C, increased CD40 expression compared to unstimulated N9 MÖ. Concomitant exposure to NotoG reduced LPS- and IFN γ -induced CD40 expression, yet slightly increased CpG- and Poly I:C-induced CD40 expression relative to the stimulant alone. **b** CpG, Poly I:C, and IFN γ , but not LPS, increased CD40 expression compared to unstimulated EOC20 MÖ. Concomitant exposure to NotoG reduced CpG-, Poly I:C, and IFN γ -induced CD40 expression relative to the stimulant alone. $n=3-6$; in triplicate. Error bars indicate the standard error of the mean. * $P < 0.05$ compared to unstimulated control (dashed line). # $P < 0.05$ compared to appropriate TLR ligand control

Fig. 4.

TLR ligand and IFN γ induced IL-6 and TNF α production by MÖ was modified by NotoG. NotoG alone exhibited no effects on either IL-6 or TNF α production. LPS, CpG, and IFN γ , but not Poly I:C increased IL-6 (**a**) and TNF α (**b**) compared to unstimulated N9 MÖ. This response was reduced by concomitant exposure to NotoG. **c** LPS, CpG, Poly I:C, and IFN γ increased IL-6 production compared to unstimulated EOC20 MÖ cells and this increase in IL-6 was reduced by concomitant exposure to NotoG. **d** In contrast, Poly I:C and IFN γ stimulated TNF α production compared to unstimulated EOC20 MÖ. Concomitant exposure to NotoG increased Poly I:C-induced TNF α production, but had no effect on IFN γ induced TNF α . $n=3-6$; in triplicate. Error bars indicate the standard error of the mean. * $P<0.05$ compared to unstimulated control (dashed line). # $P<0.05$ compared to appropriate TLR ligand control

Fig. 5.

RU486 does not affect the ability of NotoGinseng to reduce the expression of CD40 in response to LPS or IFN γ stimulation. **a** NotoG does not appear to be acting through the glucocorticoid receptor to attenuate LPS-induced CD40 expression in N9 MÖ. **b** Similarly, NotoG does not appear to be acting through the glucocorticoid receptor to attenuate IFN γ -induced CD40 expression in N9 MÖ. $n=02-3$; in triplicate. Error bars indicate the standard error of the mean. * $P<0.05$ compared to unstimulated control (*dashed line*). # $P<0.05$ compared to LPS or IFN γ . @ $P<0.05$ comparison indicated

Fig. 6.

Purified ginsenosides at comparable concentrations to those found in the whole NotoG extract exhibit differential effects on MÖ activation dependent on the stimulant. **a** The purified ginsenoside Rg1, but not Rb1 or Re, attenuated LPS-induced CD40 expression in N9 MÖ. **b** In contrast, the purified ginsenoside Re, but not Rg1 or Rb1, reduced IFN - induced CD40 expression in N9 MÖ. $n=02-3$; in triplicate. Error bars indicate the standard error of the mean. * $P<0.05$ compared to unstimulated control (*dashed line*). # $P<0.05$ compared to LPS or IFN

Table 1
Effects of NotoG on MÖ chemokine receptor expression

N9 microglia			
	CCR7 (MFI)^a	CD38 (MFI)	CCR3 (MFI)
Unstimulated ^b	152.7±1.2	17.67±2.4	19.67±0.3
NotoG	178.3±23.0 *	33.67±0.8 *	21.33±0.7
LPS	314.7±3.8 *	85.67±2.0 *	77.00±4.6 *
LPS + NotoG	322.3±33.2 *	96.33±2.3 * [#]	81.67±1.7 *
EOC20 microglia			
	CCR7 (MFI)	CD38 (MFI)	CCR3 (MFI)
Unstimulated	313.3±7.5	241.7±9.8	130.0±4.0
NotoG	294.7±6.0	231.7±7.1	121.7±2.4
Poly I:C	132.0±11.0 *	246.0±31.9	74.00±2.0 *
Poly I:C + NotoG	109.3±2.6 *	217.3±8.8	57.00±1.2 * [#]

^a mean fluorescence intensity as determined by flow cytometry

^b Microglia were either unstimulated or stimulated with LPS (100 ng/ml) or Poly I:C (250 µg/ml) in the presence or absence of NotoG (50 µg/ml) for 24 h

Results are representative of three independent experiments ($n=3$) ± standard error of the mean.

* $P<0.05$ compared to unstimulated.

[#] $P<0.05$ compared to LPS or Poly I:C only control

Table 2
Effects of purified ginsenosides on IFN γ induced nitrite production

N9 microglia	
Stimulation	Nitrite (NO₂⁻) (μM)^a
Unstimulated	2.044±0.13
NotoG	2.42±0.08 *
IFN γ	45.69±2.55 *
IFN γ + NotoG	37.29±2.42 *#
IFN γ + Re	40.59±1.60 *
IFN γ + Rb1	38.86±0.88 *
IFN γ + Rg1	40.50±0.68 *

^a micromolar (μM) concentration as determined by Griess Assay

Results are representative of three independent experiments ($n=3$) ± standard error of the mean

* $P<0.05$ compared to unstimulated.

$P<0.05$ compared to IFN γ only control