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The Role of Endophytic/Epiphytic Bacterial Constituents in the Immunostimulatory Activity of the Botanical, *Astragalus membranaceus*

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Astragalus membranaceus is a staple of Traditional Chinese Medicine being one of the oldest medicinal herbs listed in the material medica of Chinese herbal medicine. Chinese herbalists have used *Astragalus* to help the human body fight a variety of diseases. Modern herbalists utilize *Astragalus* primarily as an immunostimulant to prevent common infection and aid in the recovery following infection. Historically, the biological activities associated with *Astragalus* have been accounted for, at least in part, to several constituents present in the botanical including saponins and polysaccharides. We propose that in addition to these constituents, compounds from endophytic (or epiphytic) bacteria present in (or on) the roots of *Astragalus* may have an important biological role. Lipopolysaccharides and lipoproteins are major components of Gram-negative bacteria and highly potent activators of the innate immune response. Our data supports a direct correlation between the level of immune gene induction and the level of lipopolysaccharides/lipoproteins present in the *Astragalus* extract. We demonstrate that extracts from *Astragalus* specifically activate Toll-like and NOD-like receptors involved in the recognition and response to bacterial constituents and that removal of the lipopolysaccharide/lipoprotein from the *Astragalus* extract reduced the level of this response. The results support that many immune enhancing botanicals have established a symbiotic relationship with Gram-negative bacteria and that the immune enhancing effect of these botanical extracts on the body may not only be due to endogenous plant compounds, but endophytic (or epiphytic) bacterial components as well.

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Abbreviations: AM, *Astragalus membranceus*; TCM, Traditional Chinese Medicine; LPS, lipopolysaccharide.

Keywords: *Astragalus membranceus*, botanical, immune, lipopolysaccharide, lipoprotein, endophytic bacteria, Rhizobium

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INTRODUCTION

Astragalus membranaceus (traditionally known as Huangqi) is a traditional Chinese herb used medicinally for thousands of years for its powerful immunomodulatory effects. Typically prepared as a decoction or ethanolic extract of the root, *A. membranaceus* (AM) has been used historically to treat what Traditional Chinese Medicine (TCM) refers to as "Qi" and "Blood" deficiencies, including anemias, weakness, fever, fatigue, and uterine prolapse [1-6]. In modern clinical practice, common uses include treatment of upper respiratory infections, cardiovascular disease, cancer, diabetes mellitus, and renal disease [1,7-19]. In China, and increasingly throughout the world, AM is frequently used as an immunostimulant to prevent common infection and an immunomodulator to aid in recovery post-infection [16,20-25]. Positive therapeutic effects of AM are attributed to a wide range of antimicrobial, antiviral, hypoglycemic, cardioprotective, antioxidant, nephroprotective, and wound healing effects [7-29].

Several compounds isolated from AM have been credited with showing bioactivity i*n vitro*, *in vivo,* and in limited human clinical trials [16,26,30-32]. Over 200 plant components have been isolated and identified from AM including saponins, polysaccharides, flavonoids, alkaloids, trace elements, and amino acids [30]. Most of the attention in research has been on the activity of the polysaccharides (APS) and saponins, primarily the astragaloside saponins I-IV (AS-I, AS-II, AS-III, AS-IV). Despite decades of research, proposed mechanisms for the effects of AM remain inconclusive and many studies report conflicting evidence for immune-stimulating/pro-inflammatory effects vs. anti-inflammatory/immune-regulatory effects. APS has been credited with stimulation of macrophage maturation and phagocytosis in PMBC treated cells, increased secretion of nitric oxide (NO) and inducible nitric oxide synthase (iNOS), upregulated T-cell proliferation, and an increase in pro-inflammatory cytokines IL2, IL6, tumor necrosis factor (TNF), and interferon gamma (IFNγ) [16,30,33-37]. Conversely, the astragalosides, particularly AS-IV, have been associated with attenuation of inflammation by inhibition of toll-like receptor 4 (TLR4)/NFκB signaling pathway, reduced NO and iNOS, decreased levels of IL6, IL1β, TNFα and increased Treg cell modulation [38-42]. Although most studies continue to emphasize the dominant roles of APS and/or AS-IV in the therapeutic effects of AM, a mounting body of evidence also suggests that lipopolysaccharides (LPS, or endotoxin) and/or lipoproteins provided by Gram-negative endophytic bacteria likely play a role in its immunomodulatory activity, particularly immune stimulation [25,26,43-45].

Endophytic bacteria are symbiotic microbes found inside the tissues of all living plants that do not cause any apparent harm to the plant [46-48]. Endophytes, similar to rhizosphere microbes, interact with the plant to promote its health and development through nitrogen fixation, metabolism of waste products, and production of secondary metabolites that may be utilized by the plant and/or by humans for therapeutic benefit [48-51]. Many species of endophytic bacteria have been isolated not only from plant roots, rhizome, and root nodules, but also inter- and intracellularly from plant stems, leaves, and seeds [48]. The species and number of endophytic microbes within any given plant can vary significantly based on geography, climate, plant age, plant tissue, and other factors [52]. *Rhizobium* is an endosymbiont that has been shown to establish a relationship with AM root, and scientists have isolated 44 genetically diverse species of *Rhizobia* from 90 different geographically distinct *Astragalus* species [53,54]. Most of these *Rhizobia* are Gram-negative, non-sporulating bacilli that contain lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria helping to stabilize and protect the cell membrane [47]. LPS, in general, is highly immunogenic in humans and is able to activate macrophages and to stimulate endogenous production of pyrogens, IL1, and TNF [55-57]. LPS is also well known for acting on TLR4 receptors to stimulate a proinflammatory immune response in a host [58,59]. Alternatively, epiphytic bacteria are bacteria which live non-parasitically on the surface of a plant including the leaves, roots, flowers, buds, seeds, and fruit. These bacteria may be classified as either Gram-negative or positive often growing in aggregates or as a biofilm on the plant surface.

Recent research on another immunostimulatory herb, *Echinacea spp*. has shown that LPS and Braun-type lipoproteins from endophytic bacteria may be responsible for up to 97% of the immune stimulating activity of *Echinacea spp*. [43]. Pugh *et al.* (2013) studied the relationship between *Echinacea purpurea*'s total bacterial load, LPS content, and NF-κB activation in THP-1 macrophages and determined that the immune stimulatory activity and content of LPS was strongly correlated with the estimated total bacterial load within the plant [60]. Our previous research [25] demonstrated high levels of LPS in medicinally prepared extracts of *Astragalus*, *Echinacea spp.,* and other immunostimulatory plants compared to lower levels of LPS in *Utrica diocia* and other immunosuppressive plants. We also previously studied cytokine expression following AM treatment, both physiologically and in cell culture*,* which revealed significant increases in proinflammatory cytokines IL1α, IL1β, IL6, IL8 *in vitro*, and significant increases in IFNγ and TNFα *in vivo* [25,26]. As stated before, LPS is known to activate NFκB through stimulation of TLR4, which can lead to induction of many of these proinflammatory cytokines [30,59-61].

The purpose of our present study was to confirm the presence of immune stimulatory components, including LPS and lipoproteins likely from endophytic (or epiphytic) bacteria, in extracts of *A. membranaceus* root and to determine the relationship between these components and stimulation of TLR and NOD receptors leading to NFκB activation *in vitro*. We also sought to further examine if the *in vitro* model could be used to predict activity *in vivo* by studying the relationship between the presence of LPS/lipoproteins and levels of IFNγ and TNFα, two cytokines we previously found to be elevated in humans following oral AM administration [26].

MATERIALS AND METHODS

Cell culture: HEK-293T cells stably expressing TLR or NOD receptors were purchased from InvivoGen (293-tlr-cells). Cells were maintained in DMEM, supplemented with 4.5 g/l glucose, 2-4 mM L-glutamine, 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin and either 10mg/ml blasticidin alone or in combination with 100 mg/ml hygromycin B according to manufacturer's recommendation and incubated at 37°C with 5% CO_2 supplementation.

Botanical extract preparation: *Astragalus membranaceus* dried plant root material (origin: China) was obtained from Starwest Botanicals with documentation of authenticity. All plant material was subsequently verified by qualified botanical specialists using herbal pharmacopoeia monographs and reference keys. A voucher specimen of all plant material was deposited in our repository (SCNM #209140-31-52959). Botanical extraction protocol was based on methods done traditionally for medicinal use (personal communication, Herbal Vitality). For extraction, the botanical material was ground to a fine powder, resuspended in a 1:10 wt:vol extraction solution of 25% ethanol. The mixture was incubated for 24 hrs at room temperature with continual mixing. The solid material was removed by centrifugation at 3,000xg for 10 min and the supernatant filtered through 0.2 µM filter. The final extraction contained 10mg/ml non-volatile constituents.

NFκβ Reporter assay: Cells were transfected with pNiFty-SEAP(InvivoGen) using Lipofectamine® 2000 Reagent according to manufacturer's recommendations. Cells were allowed to recover for 48 hrs post-transfection. Cells were then stimulated/treated for 24 hrs. Changes in NFκβ expression following treatment was measured by SEAP levels in the media by using QUANTI-Blue™ (InvivoGen) absorbance according to manufactures recommendations. Control samples were treated with manufacturer's recommended agonist (InvivoGen): TLR2 and TLR2/6: heat killed *Bacteroides fragilis* (10^6 cells/ml); TLR3: poly(A:U) (1µg/ml); TLR4: LPS-B5 *E.coli 035:B5* (1µg/ml); TLR5: flagellin from *B. subtilis* (1µg/ml); TLR7 and TLR 8: single-stranded polyU naked (1µg/ml); TLR9: CpG ODN 2216 (1µM); NOD1: acetylated derivative of iE-DAP (1µg/ml); NOD2: muramyldipeptide with C18 fatty acid chain (10ng/ml).

Endotoxin removal: Ethanol was removed from botanical samples by rotary evaporation for 2 hours and the solutions and brought to the original volume with nanopure water. Botanical samples were adjusted to a pH of 7.5 with 100mM NaCl. 100ml of ToxinEraser™ Endotoxin Removal Resin (GenScript) was prepared according to manufactures recommendations and added to each treatment. Botanical samples were incubated with the resin for 24 hrs while rotating at 4°C. Resin was removed by centrifugation. Endotoxin levels were assayed using ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit and plotted against a standard curve according to manufacturer's recommendations.

Neutralization of Endotoxin by peptide blockade: Endotoxin neutralizing peptide (Sigma) was reconstituted in PBS at 1mg/ml; a final concentration was added to botanical samples at a dose of 30µg/ml of sample and allowed to incubate for 2 hrs at 4°C while rotating.

Intracellular cytokine expression: Single cell suspensions were prepared in RPM1640 + 10% heat inactivated fetal bovine serum. Cells were allowed to equilibrate for 24 hrs. $1x10^6$ cells were treated with botanical extract (with and without endotoxin removal) at a dose of 10 µg/ml for 12 hrs. At 7 hrs post-treatment, BD GolgiPlug™ was added at 1U/ml according to manufacturer's instructions. At the time of harvest, cells were pelleted by centrifugation and washed with FACS buffer and Fc Block for 20 mins at 4°C. Cells were fixed and permeabilized with BD permeabilization solution for 20 mins at 4°C, followed by washing of the cells two times in 1× BD Perm/Wash™ buffer. Staining for intracellular cytokines was conducted with anti-human IFNγ or anti-human TNFα (BD Pharmingen) by incubating cells with antibodies at 4°C for 30 mins in the dark. Cells were then washed two times with 1× BD Perm/Wash™ buffer. Stained cells were then immediately acquired on a BD LSR II Fortessa flow cytometer and analyzed using FlowJo software.

RESULTS

Toll-like receptors (TLRs) make up a class of proteins that play a fundamental role in the recognition of pathogen-associated molecular patterns (PAMPs) expressed on infectious organisms [59]. Although various TLRs detect a wide variety of microbial components, all TLR signaling pathways culminate in activation of the transcription factor, NF-κB, which controls the expression of an array of inflammatory cytokine genes that make up the innate

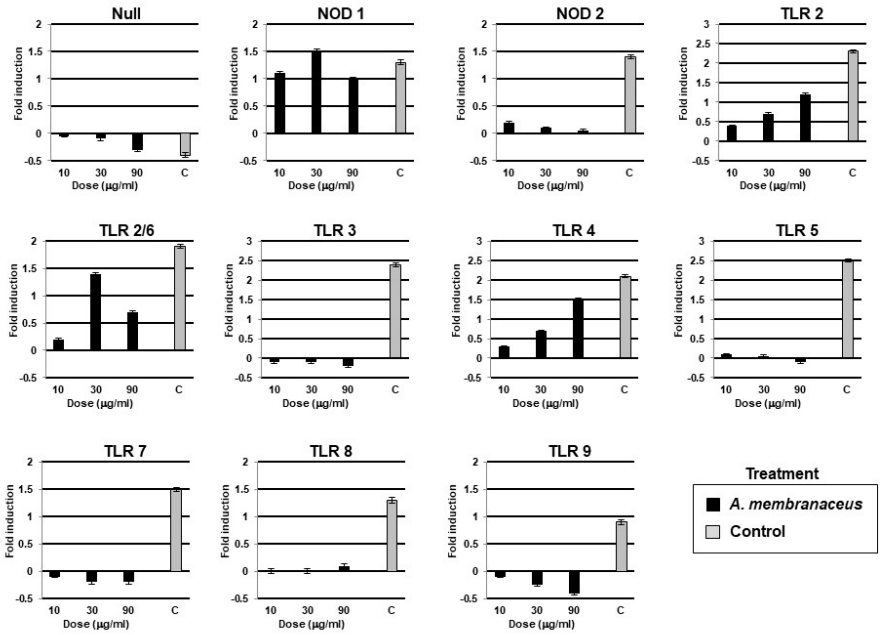

Figure 1. TLR/NOD receptor activation by *A. membranaceus*. HEK293 cells stably expressing the indicated human TLR/NOD gene were treated with AM extracts at 10, 30, and 90 µg/ml. Control samples (C) were treated with manufacturer's recommended receptor agonist. NF-κB activation was evaluated by expression of the NF-κB inducible reporter gene and secretion of SEAP. Error bars indicate the standard deviation from three separate trials.

immune response [56,59]. Similarly, nucleotide-binding and oligomerization domain (NOD)-like receptors are pattern-recognition receptors similar to TLRs. While TLRs are transmembrane receptors, NLRs are cytoplasmic receptors that play a crucial role in the innate immune response [62]. Of the TLR protein family, TLR4 is the most acknowledged member for bacterial LPS recognition and LPS-mediated inflammatory responses [58,59]. TLR2 and TLR6 form heterodimers and are associated with bacterial lipoprotein recognition [42,58,59]. Peptidoglycan from Gram-negative bacterial cell wall has been associated with activation of NOD1 proteins [62]. In order to study the activation of TLR and NOD receptors by suspected LPS present in the *A. membranaceus* extract, HEK293T cells were obtained that contained either no receptor (Null cell) or stably expressing NOD or TLR receptors, including the following: NOD1, NOD2, TLR2, TLR3, TLR2/6, TLR4, TLR5, TLR7, TLR8, and TLR9. HEK293T cells expressing the indicated NOD/TLR genes and the HEK293T null cell were transiently transfected with the SEAP plasmid and subsequently treated with AM extract or a manufacturer's recommended known agonist for the receptor (control). Concentrations of AM extract were based on total non-volatile components present in the extract. Changes in NFκB activation following cell treatment were measured by quantifying SEAP (secreted embryonic alkaline phosphatase) levels in the media. Control treatment of all NOD/TLR expressing cells resulted in induction of NFκB, compared to the null cell, in which NFκB was not induced (Figure 1). Treatment of the NOD/TLR cells with AM extract resulted in activation of NF-κB in cells expressing NOD1, TLR2, TLR2/6, and TLR4 (Figure 1). Conversely, cells expressing NOD2, TLR3, TLR5, TLR7, TLR8, and TLR9 did not lead to activation of NFκB following administration of AM (Figure 1). TL4 has been associated with stimulation by bacterial LPS, whereas TLR2 has been associated with stimulation by bacterial lipoproteins, both leading to subsequent induction of the NF-κB cascade [58,59,63]. Our results show that activation of the TLR4 and TLR2 cells was dose dependent with levels comparable to the control sample (Figure 1). TLR2 typically dimerizes with TLR6 to mediate the cellular response to bacterial lipoproteins [63], so a single cell expressing both TLR2/6 was tested, which also revealed positive induction by AM (Figure 1). At the highest dose of AM (90 µg/ml) a reduction in activation was observed in the TLR2/6 cells resulting in a decrease in SEAP activity compared to the 30 µg/ml dose (Figure 1). This reduction in activity could be due to the presence of inhibitory compounds also present in the extract, potentially including other lipoproteins which have been shown to attenuate TLR2 activation [60,64]. Both NOD1 and NOD2 proteins recognize specific peptidoglycan constituents of bacterial cell walls, however, NOD1 has been associated with stimulation by Gram-negative bacteria, whereas NOD2 is generally associated with

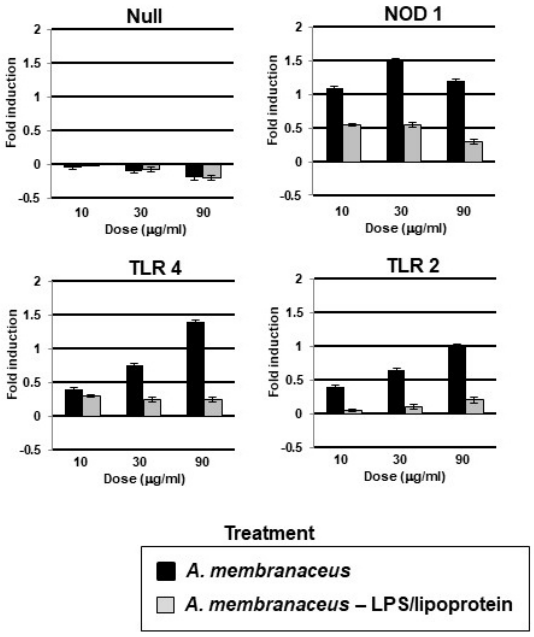

Figure 2. Inhibition of TLR/NOD activation by *A. membranaceus* following endotoxin removal. HEK293 cells stably expressing the indicated human TLR/NOD gene were treated with AM extracts or AM extracts following LPS (endotoxin)/lipoprotein removal at 10, 30, and 90 µg/ml. NF-κB activation was evaluated by expression of the NF-κB inducible reporter gene and secretion of SEAP. Error bars indicate the standard deviation from three separate trials.

stimulation by either Gram-positive or negative bacteria [62]. NOD1 cells were highly activated by AM (even at the lowest dose), whereas NOD2 cells were not activated, further supporting immune activation by bacterial constituents (Figure 1). Again, a partial inhibition in activation was observed following treatment of the NOD1 cells at the highest dose of AM extract. As previously stated, TLR3, TLR5, TLR7, TLR8, and TLR9 were not induced following administration of AM (Figure 1). Most of these receptors have not been shown to respond to LPS, lipoproteins, or peptidoglycan cell wall components. TLR3 is generally associated with activation by viral dsRNA, and similarly, TLR5 is associated with the recognition of bacterial flagellin, a virulence factor found in certain species of Gram-positive or Gram-negative bacteria that contain flagella [59,65]. TLR7 and TLR8 primarily recognize viral ssRNA molecules, and TLR9 has been identified as being able to sense DNA-containing viruses and unmethylated CpG dinucleotides in bacterial DNA [59,66-68]. Since most of these TLRs are involved in recognizing viral constituents (except for TLR 5), it is likely that the AM did not contain viral pathogens or that the PAMPs for these TLRs were not extracted utilizing the 25% ethanol extraction conditions.

In order to confirm the role of LPS and lipoproteins as active constituents in the induction of cellular NF-κB expression, endotoxin/LPS removal was performed on AM extracts. Endotoxin removal is a critical procedure performed in many recombinant protein preparations of drugs, injectables, and other biologic products, to reduce the risk of LPS-related shock, tissue injury, or other serious side effects associated with the presence of endotoxin [69]. In this experiment, endotoxin/LPS removal was performed as described using a polymyxin B based affinity resin and column filtration. In addition, lipoproteins have also been shown to bind to a polymyxin B resin and were likely removed from the extract during this process [70]. Endotoxin levels were undetectable following this procedure (data not shown). Transfected HEK293T cells containing NOD1, TLR2, TLR4, and the null cell were treated with the AM extract (with and without endotoxin removal), and were evaluated for NF-κB activation. The null cell, as in the first experiment, was not induced by either AM extract or AM extract without endotoxin (AM-LPS). Conversely, NOD1, TLR2, and TLR4 cells were all activated by AM extract, to a similar degree as in the first experiment (Figure 1), but this level of activation was greatly inhibited when treated with the AM-LPS extract (Figure 2). As mentioned before, TLR4 proteins are associated with recognition of bacterial LPS, whereas TLR2 is associated with recognition of lipoproteins and NOD1 is associated with recognition of peptidoglycan from Gram-negative bacterial cell wall [58,59,62]. Although the endotoxin removal assay was designed to remove

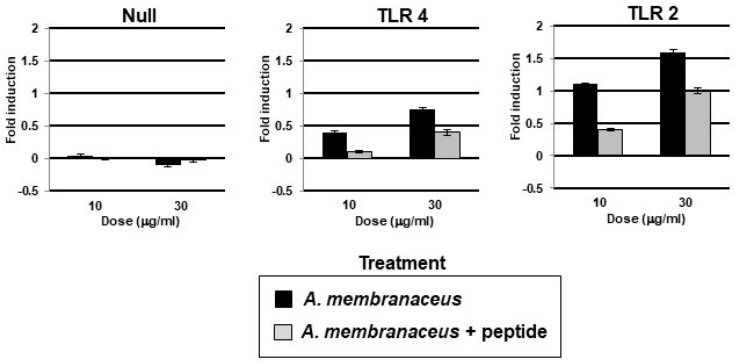

Figure 3. Inhibition of TLR activation by *A. membranaceus* following endotoxin inhibition. HEK293 cells stably expressing the indicated human TLR/NOD gene were treated with AM extracts or AM extracts treated with endotoxin neutralizing peptide at 10 and 30 µg/ml. NF-κB activation was evaluated by expression of the NF-κB inducible reporter gene and secretion of SEAP. Error bars indicate the standard deviation from three separate trials.

LPS, it can also bind lipoproteins. It is likely that the LPS and/or lipoproteins were structurally associated with the bacterial peptidoglycan components leading to the removal of these components during the column filtration process and the subsequent reduction in NOD1 activation seen in Figure 2. These results support that LPS and lipoproteins present in the AM extract, and possibly peptidoglycan in the case of NOD1, was likely responsible for inducing NF-κB in the TLR4 and TLR2 expressing cells.

Since it is possible that column filtration may remove additional components other than just LPS/lipoproteins, the role of the LPS/lipoproteins as the active constituents in the induction of TLR receptors was confirmed by the addition of endotoxin peptide inhibitor. In this assay, an endotoxin neutralizing peptide was added to AM extract to inhibit the activity of the LPS. Transfected HEK293T cells expressing TLR4 or TLR2 and the null cell were treated with the AM extract, or the AM extract incubated with endotoxin neutralizing peptide (AM+peptide). As expected, the null cell was not induced by either the AM extract or AM+peptide (Figure 3). TLR2 and TLR4 cells were both activated by the standard AM extract, to a similar degree as previously observed (Figure 3). Activation of the TLR2 and TL4 cells was greatly inhibited by treatment with AM+peptide as compared to activation by AM extract alone (Figure 3). Although a full inhibition in the activation of the TLR2 and TLR4 cells was not observed, the level of inhibition was substantial and consistent. We believe stoichiometrically we were not able to add enough peptide to bind and neutralize all the LPS present in the extract. These results confirm our previous results observed in Figures 1 and 2, supporting the presence and role of LPS in the activation of TLR4. For TLR2, the endotoxin neutralizing peptide was also likely able to block the activity of bacterial lipoproteins leading to the inhibitory effect observed.

Figures 1-3 demonstrate that LPS and lipoproteins are likely present in AM extracts and that these constituents bind to TLRs initiating the NFκB cascade. Although these studies show there is a clear cellular effect, we also sought to demonstrate whether the LPS/lipoproteins present were responsible for any physiological effects. Previous studies on *Astragalus* have shown that treatment of human PMBCs (peripheral blood mononuclear cells) with hydroethanolic extracts of AM induced the maturation of monocytes/macrophages and the release of multiple immunostimulatory and proinflammatory cytokines, such as IL1, IL2, IL6, TNFα, and IFNγ [25]. Our previous research on the physiological response of AM showed that circulatory TNFα and IFNγ levels were significantly increased in human serum following *in vivo* administration of AM [26]. PMBCs are a mixture of mostly lymphocytes (T-cells, B-cells, and natural killer cells) as well as monocytes, macrophages, and dendritic cells, that are used frequently in immunological research cell-culture studies [71-73]. Based on our previous research findings, we chose to look at the expression of TNFα and IFNγ in human PMBCs following treatment with AM, with and without LPS/lipoprotein removal. Human PMBCs were treated with either AM extract or AM extract that was column filtered to remove endotoxin (AM-LPS). Levels of TNFα and IFNγ expression were measured using anti-human TNFα and anti-human IFNγ antibody staining followed by flow cytometry analysis. Treatment of PMBCs with vehicle (25% ethanol) showed no detectable IFNγ expression (Figure 4A). Treatment with AM resulted in the detection of two cell populations, with one group expressing high levels of IFNγ activity, indicating production of IFNγ by some cells in the PMBC population (Figure 4A). When the LPS/lipoproteins were removed from the AM extract and the cells treated, IFNγ expression was not detected within the cell population

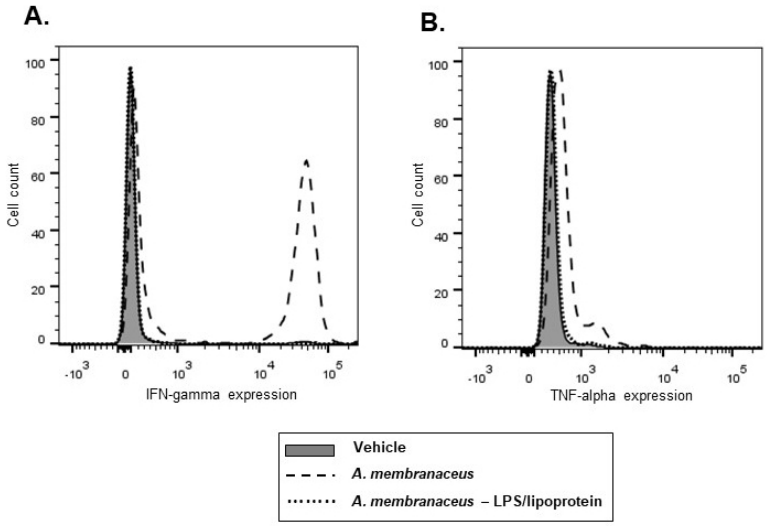

Figure 4. Activation of human PBMC cells by *A. membranaceus*. PBMCs were treated with vehicle (25% ethanol), AM extract, or AM extract following endotoxin removal at a dose of 10 µg/ml. At the time of harvest, cells were fixed, permeabilized, and probed with anti-human IFNγ Part A) or anti-human TNFα Part B). Stained cells were acquired on a BD LSR II Fortessa flow cytometer and analyzed using FlowJo software. Vehicle treated cells are shown in gray; AM extract treated cells are shown in large dashed lines; AM extracts with endotoxin removed are shown in small dashed lines.

producing a similar profile as the vehicle control sample (Figure 4A). Similarly, although less prominently, treatment of PMBCs with AM resulted in shift in the cell population leading to the production of TNFα compared to the vehicle control (Figure 4B). However, when these cells were treated with AM depleted of LPS/lipoproteins, no TNFα expression was detected (Figure 4B). These findings confirm what our previous studies demonstrated and support the potential physiological role of LPS/lipoproteins in stimulating production of IFNγ and TNFα in human PMBCs following treatment with AM extracts.

DISCUSSION

The results presented strongly suggest the presence of bacterially-derived LPS/lipoproteins in AM root extracts and that LPS/lipoproteins contribute to purported cell activation and stimulation of cytokine expression *in vitro*. Although the majority of studies on *Astragalus* support polysaccharides (APS) and astragalosides as the predominant active constituents in AM [30], our results demonstrate that LPS/lipoproteins are also likely present in medicinally prepared extracts of AM and should be considered a probable active component in the immunostimulatory activity of the plant. Validation and confidence in the results obtained from the NFκβ Reporter assay was supported in our results where (1) the activation of specific bacterial TLRs and NOD receptors (TLR2, TLR4, and NOD1) was observed after treatment with AM extracts (2) the activity of TLR2, TLR4, and NOD1 was inhibited following removal of LPS/lipoproteins by column-filtration or by an endotoxin binding peptide, and (3) TNFα and IFNγ expression in PBMCs was likely dependent on the presence of LPS/lipoproteins in the AM extract. Although these assays were done in a cell culture system, previous results *in vivo* support the induction of TNFα and IFNγ following the oral administration of AM and therefore may support a role of LPS/lipoproteins in these physiological effects [26].

Complementary and alternative medicine (CAM) has been a staple in Eastern medicine for millennia and is still thriving in places like China where the adoption of Western medical practices in recent decades has resulted in the creation of integrative Chinese and Western medicine (ICWM) in clinics and hospitals [74]. CAM continues to garner attention in Western medicine and throughout the developed world for its perceived natural and effective prophylactic, adjunctive, and interventional benefits [44,75,76]. The most recent survey on CAM users in the US by the National Institutes of Health reported that nearly 4 out of 10 adults used some type of CAM in the past year, and that, of the various types, non-vitamin, non-mineral products like *Echinacea* (37.2% of children and 19.8% of adults using non-vitamin, non-mineral products) were the most common [76]. A systemic review by Posadzki *et al.* (2013) also found that herbal medi-

cine was the most popular CAM used by patients and consumers in the UK during the past decade [75]. Due to the increasing popularity of CAM, particularly herbal medicine, it is imperative that we better understand the constituents and therapeutic mechanisms of these plants in order to create proper standards and effective clinical protocols.

The results of this study suggest that endophyte/epiphyte-derived LPS/lipoproteins within AM root are probable active constituents responsible for the immunostimulatory effects of *Astragalus*. Of approximately 300,000 plant species identified so far on earth, at least one species of endophyte has been found in every single plant [46-48]. Over the past 20 years, interest in plant-microbe interactions has been increasing, in part due to the use of endophytes as valuable biocontrol agents, including the genetic engineering of antipest protein expression and biodegradation of soil pollutants [52]. Different endophytic species have been found to preferentially inhabit different plant tissues and tend to vary widely based on plant species, age, geography, climate, and season of harvest [45,46,52,77]. Diverse rhizobia (endophytes residing in root or stem nodules) belonging to the genera *Rhizobium, Mesorhizobium, Ensifer,* and *Bradyrhizobium* have all been isolated from *Astragalus spp.* grown in different geographic regions throughout Europe, North America, and Asia [78-82]. A study by Chen *et al.* (2015) reported isolation of 78 different bacterial strains from the root nodules of 12 *Astragalus* species grown in different geographic regions of northwestern China [83]. The dried root plant material used in our study was sourced from a single origin in China and thus only represents one geographic origin; however, most *Rhizobia* identified in *Astragalus* to date are Gram-negative, non-sporulating bacilli that contain LPS/lipoproteins [47,53,83]. Therefore, it is likely that LPS/lipoproteins would be present in extracts prepared from most species of *Astragalus*, regardless of plant origin. Further studies are needed to identify the full spectrum of endophytic (and epiphytic) bacteria and the LPS/lipoproteins in AM based on plant species and geographic origin.

This study emphasizes the probable role of pro-inflammatory LPS/lipoproteins from endophytic/epiphytic bacteria in the induction of an early acute immune response following AM treatment. This supports previous research where Pugh *et al*. (2008) attributed the majority of immune enhancing botanicals' *in vitro* macrophage activating properties to the presence of both LPS and bacterial lipoproteins acting through TLR4 and TLR2, respectively [43]. In addition, extracts from specific botanicals, including *Angelica sinensis, Ashwaganda,* and *Echinacea purpurea* have been shown to have immune stimulatory activity linked to plant-associated bacteria [44,84-85]. Furthermore, there may be other synergistic compounds and other mechanisms involved in AM's immunomodulatory activities. Recent research by Maggini *et al.* (2017) on *Echinacea purpurea (L.)* found that bioactive secondary metabolites produced by normal plant-endophyte interactions may be correlated with the levels and therapeutic activity of these same bioactive compounds seen *in vivo* [50]. Many endophytes are known to have positive symbiotic relationships with their hosts, including the production of secondary compounds that aid the plant in its growth, health, and survival [49-51,86-89]. It is possible that these bioactive compounds, in addition to LPS/lipoproteins, could play a role in the therapeutic activities of AM *in vivo*.

Medicinal plants are often difficult to study because of subtle variations in plant material and the presence of multiple bioactive compounds operating synergistically or antagonistically *in vivo* [43,44,50,90]. Previous research on *Astragalus* largely supports APS and AS-IV as the primary constituents in AM. AS-IV is even used as a vital marker for quality control of plant material in Chinese Pharmacopoeia [30]. Despite the vast amount of literature, and much like the current body of evidence surrounding *Echinacea*, there remains conflicting evidence as to whether therapeutic immunomodulatory effects of AM are anti-inflammatory, immunostimulatory, or a combination of both. In our present study, and in support of current LPS/lipoprotein results observed for *Echinacea,* AM LPS/lipoprotein was shown to be proinflammatory and to stimulate NFκB pathways via TLR4 and TLR2/6 and to upregulate TNFα and IFNγ [44,45,50,60,77]. TNFα is an acute phase protein that promotes the inflammatory response during the innate immune response [91]. IFNγ is involved in macrophage activation and upregulation of antiviral and antimicrobial effector molecules [91]. Both TNFα and IFNγ stimulate the release of nitric oxide from macrophages, and together can synergistically induce other NF-κB responsive genes [92]. Conversely, AS-IV has been shown in multiple studies to be anti-inflammatory and to attenuate LPS-induced inflammatory cytokines by inhibiting TLR4 and NFκB [38-42]. Considering the complex nature of medicinal plants and the human immune system, it is possible that both anti-inflammatory and immunostimulatory mechanisms are at play following the administration of AM.

In conclusion, though these studies do not provide conclusive proof that LPS/lipoproteins cause the immunostimulatory activity seen following administration of *Astragalus membranaceus*, they strongly support the presence of endophytic (or epiphytic) bacteria and LPS/lipoproteins in medicinally prepared extracts of AM and suggests a correlation between the presence of LPS/lipoproteins and the activation and mobilization of the innate immune system.

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