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Cell-line based assay for the toxicity/benefit analysis of lipopolysaccharides in plants

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Abstract

There are many immune-boosting medicinal plants that can potentially activate innate immune cells. Recent studies indicate that the active factors of some immune-boosting plants are lipopolysaccharides (LPSs) of plant-associated bacteria. However, little is currently known about the potential risk and benefit of LPSs in medicinal plants. To facilitate their characterization, we established a simple cell-line based assay that can be used to screen the toxicity and benefit of LPSs in medicinal plants. The assay can distinguish endotoxic diphosphoryl lipid A (DPL) from beneficial monophosphoryl lipid A (MPL), which is a clinically used immunological adjuvant for vaccines. The established assay was used to characterize commercial supplements of Ashwagandha, which was shown to contain immunostimulatory LPSs. The study revealed that Ashwagandha activates macrophages in a manner similar to MPL. The current finding underscores the importance of further studies to characterize the LPSs in immune-boosting medicinal plants.

Graphical Abstract

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting Information: Experimental procedures may be found online in the supporting information tab for this article.

A simple and inexpensive assay was developed to characterize the safety and benefit of immunomodulatory lipopolysaccharides that have been recently found in medicinal plants. The assay revealed that lipopolysaccharides in Ashwagandha, an Ayurvedic medicinal plant, activate macrophages in a manner similar to monophosphoryl lipid A, a clinically used vaccine adjuvant.

Keywords

MyD88; TRIF; Lipopolysaccharides; MPL; Ashwagandha

Various immune-boosting medicinal plants are used to treat infectious diseases throughout the world. For example, *Echinacea* is a popular immune-boosting remedy in Europe and North America to alleviate cold symptoms (Woelkart, Linde, & Bauer, 2008). Oriental herbal medicines, including Japanese Kampo, have various herbal formulations, such as Juzen-taiho-to (JTT) (Yamada & Saiki, 2005), to boost immune functions. Similarly, Ashwagandha (*Withania somnifera*) of Ayurvedic Medicine is known for the immunostimulatory activity as well as for other therapeutic effects (Ziauddin, Phansalkar, Patki, Diwanay, & Patwardhan, 1996). Although these immune-boosting plants are known to activate the innate immune cells, such as monocytes and macrophages, the molecular factors responsible for the activity were unknown for a long time.

Recent studies revealed that the immunostimulatory factors in *Echinacea* and JTT were lipopolysaccharides (LPSs) of plant-associated Gram-negative bacteria (Haron et al., 2016; Kalpana et al., 2019; Montenegro et al., 2015; Pugh, Jackson, & Pasco, 2013; Pugh et al., 2008; Todd et al., 2015). These findings opened a possibility that the immunostimulatory activity of other medicinal plants might also arise from LPSs. To explore this possibility, we examined Ashwagandha, an Ayurvedic medicinal herb that is gaining popularity in the United States (Smith, Kawa, Eckl, Morton, & RStredney, 2018). Human monocytic cell line (THP-1 cells) was treated with Ashwagandha and subjected to reverse transcription

quantitative PCR (RT-qPCR) of intercellular adhesion molecule 1 (ICAM-1), which is a biomarker of monocyte activation (Takaoka et al., 2014). Ashwagandha indeed activated THP-1 monocytes as indicated by the induction of ICAM-1 mRNA (“Ash” in Figure 1a,b). However, when Ashwagandha was treated with an agarose-based affinity resin containing polymyxin-B (Detoxi-Gel™) (Figure 1a), which depletes LPSs (Issekutz, 1983; Talmadge & Siebert, 1989), the activity disappeared (“Ash PmxB” in Figure 1b). On the other hand, the treatment of Ashwagandha with agarose-resin (a mock-treatment) did not alter the activity (“Ash Mock” in Figure 1b). Taken together, these results indicated that the immunostimulatory factors of Ashwagandha are also LPSs of plant-associated bacteria.

The presence of immunostimulatory LPSs in medicinal plants raises a new safety concern because LPSs can be toxic. In fact, LPSs of human pathogens, such as *Salmonella minnesota* (occasional human pathogen), are so-called endotoxins due to their potent pro-inflammatory toxicity (Bohannon, Hernandez, Enkhbaatar, Adams, & Sherwood, 2013). Their endotoxicity arises from the lipid A moiety (Figure 2a), which binds to the receptor complex of myeloid differentiation protein-2/toll-like receptor 4 (MD-2/TLR-4) to trigger strong pro-inflammatory responses through the pathway mediated by myeloid differentiation primary response 88 (MyD88) (Figure 2b). Although inflammation is an important part of immunological defense against infection, endotoxins can over-stimulate the MyD88 pathway to cause serious tissue injuries.

In addition to the pro-inflammatory MyD88 pathway, endotoxins also activate another pathway, which is mediated by Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) (Figure 2b). The TRIF pathway is known to play an important role in protective immunity against bacterial and viral infections (Mata-Haro et al., 2007; Pryke et al., 2017; Sotolongo et al., 2011). This ability of endotoxins to promote protective immunity can be harnessed to generate safe immunostimulatory agents. The most successful example is monophosphoryl lipid A (MPL) (Figure 2d), which was derived from an endotoxic diphosphoryl lipid A (DPL) of *S. minnesota* (Figure 2c). Removal of a phosphate and an acyl group from DPL gives MPL, which activates the MyD88 pathway only modestly and thus is devoid of pro-inflammatory toxicity. Yet, MPL retains the ability to activate the TRIF pathway. As such, MPL can be used to safely stimulate the innate immune system to promote protective immunity. In fact, MPL has been used widely as an immunological adjuvant to boost the efficacy of clinical and experimental vaccines (Dubensky & Reed, 2010; Harper et al., 2006).

At present, little is known about the immunostimulatory LPSs that have been detected in some medicinal plants. Their effects on the pro-inflammatory MyD88 pathway have to be characterized to evaluate their safety. In addition, their effects on the TRIF pathway should also be examined to determine their ability to promote protective immunity. The existing assays to detect the MyD88 and TRIF pathways, however, use primary macrophages or animal models (Mata-Haro et al., 2007), which are difficult to handle and costly for screening of many plant samples. A simple cell-line based assay to detect the two pathways, if developed, would greatly facilitate the evaluation of LPSs in medicinal plants. Here, we present a simple and inexpensive cell-line based assay to quantify the activation of both

MyD88 and TRIF pathways. Furthermore, the utility of the assay is demonstrated by the characterization of commercial Ashwagandha supplements.

In order to establish a simple cell-line based assay to detect the MyD88 and TRIF pathways, we initially examined THP-1 monocytic cells. It turned out, however, that, while the activation of the MyD88 pathway could be readily detected, the TRIF pathway activation was hardly detectable in THP-1 cells (data not shown). As such, we then attempted to differentiate THP-1 cells by phorbol 12-myristate 13-acetate (PMA), which has been used to make THP-1 cells more responsive to cytokines, such as tumor necrosis factor (Lund, To, O'Brien, & Donnelly, 2016). Treatment with 25 nM PMA for 48 hours, followed by a 24-hour recovery period in the absence of PMA, elicited differentiation of THP-1 cells into a macrophage-like phenotype (Figure 3). The differentiated THP-1 cells were treated with the endotoxin DPL and subjected to RT-qPCR analysis of chemokine ligand 5 (CCL5) mRNA, which is a biomarker of the TRIF pathway activation; CCL5 is also known as RANTES (regulated on activation, normal T cell expressed and secreted). The RT-qPCR analysis revealed that DPL clearly activated the TRIF pathway as evidenced by the nearly 10-fold induction of CCL5 over the DMSO vehicle control (Figure 3). The DPL treatment also potently stimulated the pro-inflammatory MyD88 pathway, as indicated by ca. 1000-fold induction of interleukin-6 (IL-6) mRNA, a biomarker of the MyD88 pathway (Figure 3). The observed MyD88/TRIF profile of DPL was consistent with the reported strong MyD88-bias of endotoxins (Mata-Haro et al., 2007). MPL, on the other hand, elicited very different responses. MPL activated the MyD88 pathway much more weakly than DPL, as indicated by the ~7-fold induction of IL-6 (Figure 3). This result is consistent with the fact that MPL is devoid of pro-inflammatory toxicity. MPL, however, clearly activated the TRIF pathway as indicated by the ~4-fold induction of CCL5. Thus, the MyD88/TRIF profile of MPL was also consistent with the previous report (Mata-Haro et al., 2007). Taken together, these results indicated that the PMA-differentiated THP-1 cells could serve as a simple and economical system for the toxicity/benefit analysis of LPSs in medicinal plants.

Having established the simple assay to detect the two pathways, we decided to start characterizing medicinal plants known to contain immunostimulatory LPSs. As a first step, we examined two commercial supplements of Ashwagandha, namely, Daily Nutra™ KSM-66® Ashwagandha (AS1) and Organic India™ Ashwagandha (AS2). The PMA-differentiated THP-1 cells were treated with these supplements and subjected to the RT-qPCR analyses of IL-6 and CCL5 to quantify the activation of the MyD88 and TRIF pathways, respectively. Unlike DPL (the endotoxin control), which exhibited a strong MyD88 bias, both Ashwagandha supplements exhibited more evenly balanced MyD88/TRIF activation profiles, which were similar to the profile of MPL (Figure 4). The results indicated that Ashwagandha supplements could safely stimulate innate immune cells without eliciting strong pro-inflammatory responses.

The current study demonstrates that the PMA-differentiated THP-1 cells serve as a simple system to detect the LPS-induced activation of both MyD88 and TRIF pathways. Unlike primary cells or animal models, which are expensive and difficult to handle, THP-1 is an immortalized cell line that is readily available and reproducibly differentiated by PMA into a macrophage-like phenotype. The PMA-differentiated THP-1 cells can distinguish the strong

MyD88-bias of endotoxin (DPL) and more balanced MyD88/TRIF profile of MPL. Thus, the system serves as a simple platform to screen the safety and benefit of LPSs in medicinal plants. This is important because, while the LPSs of human pathogens (endotoxins) have been extensively studied, little is currently known about the LPSs in medicinal plants. Furthermore, the assay can also serve as a powerful tool to screen the safety and benefit of environmental LPS and synthetic analogs of lipid A.

Although the current study used IL-6 and CCL5 to detect the activation of the MyD88 and TRIF pathways, respectively, other genes downstream of these pathways could also be analyzed. For example, the MyD88 activation could also be monitored by cyclooxygenase 2 (COX-2) mRNA in the PMA-differentiated THP-1 cells. DPL caused more than 1000-fold induction of COX-2, whereas MPL induced COX-2 much more modestly (~10-fold) (Figure S1). However, detection of the TRIF pathway is more difficult. In our hands, CCL5 gave the most reproducible results. Interferon gamma-inducible protein 10 (IP-10), which is also regulated by the TRIF pathway, could not be detected in the PMA-differentiated THP-1 cells.

It is, however, also important to note that the assay, as presented in this manuscript, is for the initial screening of plant samples. Admittedly, the MyD88/TRIF profile detected by the assay is preliminary in nature, because the assay is based on an immortalized cell-line and measures only two cytokines. There is also a possibility that compounds other than LPS in the sample can modulate the MyD88 and TRIF pathways. As such, once plant samples of interest are identified, their immunological activities as well as the factors responsible for the activities need to be further characterized.

The current study also suggests that the commercial Ashwagandha supplements are similar to MPL in terms of IL-6 and CCL5 induction. Although more comprehensive studies are needed to fully define the similarities and differences between the LPSs in Ashwagandha and MPL, the current finding opens a possibility that Ashwagandha and other immune-boosting plants may serve as an untapped source of new LPSs with therapeutic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The immunostimulatory activity of Ashwagandha (Ash) is diminished by polymyxin-B treatment. (a) Ash treated with polymyxin-B (Ash PmxB) and agarose (Ash Mock), which examined non-specific binding, were prepared. (b) ICAM-1 RT-qPCR of the samples revealed that the polymyxin-B treatment significantly reduced the activity of Ash compared to the mock treatment. The mock treatment did not significantly alter the activity compared to the Ash sample with no treatment. THP-1 cells were treated with DMSO (vehicle control), Ash samples (5 μ g/mL), or LPS (0.2 ng/mL, positive control) for 4 h. Each sample was analyzed in triplicate. RQ (Relative Quantitation): fold change. NS (not significant)

Figure 2.

Immunological pathways activated by lipopolysaccharide (LPS) and lipid A (LA). (a) General structure of LPS. The polysaccharide (Core and O-antigen) moiety is highlighted in blue. The LA moiety is shown in red. (b) A simplified overview of the TLR4/MD2 receptor activation by LPS. LPS is transferred to the TLR4/MD2 receptor complex by LPS-binding protein (LBP) and CD14. The LA moiety of LPS is recognized by TLR4/MD2 to trigger the activation of MyD88 and TRIF pathways, which are associated with inflammation and protective immunity, respectively. The activation of the MyD88 and TRIF pathways can be monitored with the downstream cytokine genes, such as IL-6 and IFN- γ for MyD88 and CCL5 and MCP-1 for TRIF. TRAM is TRIF-related adaptor molecule. (c) The diphosphoryl lipid A (DPL) of *Salmonella minnesota* R595 (pathogenic bacteria). It preferentially activates the MyD88 pathway, which triggers strong pro-inflammatory responses. Removal of the functional groups highlighted in red gives monophosphoryl lipid A (MPL), a clinical vaccine adjuvant, (d) Structure of MPL. MPL is a much weaker activator of the MyD88 pathway than *S. minnesota* LA, but it retains the ability to activate the TRIF pathway.

Figure 3.

A cell-line based assay for the determination of MyD88/TRIF bias. The MyD88 (in red) and TRIF (in green) pathways were observed by qRT-PCR of IL-6 and CCL5, respectively, using the PMA-differentiated THP-1 cells (THP-1 cells were differentiated by 25 nM PMA for 48h, followed by a recovery period of 24 h in culture in the absence of PMA). DMSO (vehicle control), MPL (monophosphoryl lipid A, a clinical vaccine adjuvant, 5 µg/mL) and DPL (diphosphoryl lipid A of *E. coli*, endotoxic, 5 µg/mL). Each sample was analyzed in triplicate. RQ (Relative Quantitation): fold change. The assay can reproducibly detect the notable difference in pro-inflammatory effect between MPL and DPL: MPL elicited 7-fold induction of IL-6 over DMSO control, whereas DPL caused about 1,000-fold induction of IL-6.

Figure 4.

The MyD88/TRIF balance of commercial Ashwagandha supplements. qRT-PCR assays indicate that Ashwagandha supplements (AS1 and AS2) do not exhibit the strong MyD88 bias of DPL. Rather, they resemble MPL in terms of the MyD88/TRIF profile. PMA-differentiated THP-1 cells were treated with AS1_250 and AS2_250 (250 $\mu\text{g/mL}$), AS1_500 and AS2_500 (500 $\mu\text{g/mL}$), MPL and DPL (5 $\mu\text{g/mL}$, positive control), or DMSO (vehicle control) for 4 h. Although Ashwagandha is consumed in water in the practice of herbal medicine, this study used their DMSO solutions because the MPL and DPL controls were not soluble in water (they are soluble in DMSO). Each sample was analyzed in triplicate. RQ (Relative Quantitation): fold change.