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Original Research

Effect of Inositol Hexaphosphate on Lipopolysaccharide-Stimulated Release of TNF-α from Human Mononuclear Cells

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Abstract

Inflammatory cytokines, including TNF-α, are produced by mononuclear leukocytes in response to numerous agents, such as microorganisms and microbial products, e.g., lipopolysaccharides (LPSs). We studied the modulation of LPS-induced release of TNF-α from human mononuclear cells by inositol hexaphosphate (IP6). This naturally occurring phytochemical, abundantly present in a regular diet, possesses several pharmacological activities beneficial for human health involving anticancer function and the ability to enhance the immune system. The present study on the effect of IP6 on the challenge of host defense system in cases of endotoxemia adds more to physiological importance of IP6 in terms of its immunomodulatory activity. Incubation of cells with IP6 alone (up to 250 μM) had no effect upon the basal secretion of TNF-α, whereas at higher doses it acted as an agonist by up-regulating the cytokine release. Incubation of cells with IP6 prior to LPS challenge resulted in differential effects which were dependent on triggering LPS. The response of cells to LPS from *Desulfovibrio desulfuricans* and *Escherichia coli* was diminished by IP6. Cell priming by IP6, resulting in up-regulation of TNF-α release was observed with *Salmonella minnesota* LPS stimulation. These results indicate that IP6 may exert immunoregulatory effects on mononuclear cell function and control their level of activation states.

Keywords: inositol hexaphosphate, IP6, phytic acid, bacterial lipopolysaccharides, TNF-α, mononuclear cells, immunomodulation

Introduction

Inositol hexaphosphate (IP6 or phytic acid) is a naturally occurring polyphosphorylated carbohydrate, found in many plant sources and in certain high-fiber diets such as wheat bran, legumes, nuts and oil seeds [1]. It is also ubiquitous in eukaryotic species [2], and is typically the most abundant inositol phosphate found in eukaryotic cells with its concentrations ranging from 10 μM to 100 μM [3]. As an ingredient of dietary fiber IP6 is physiologically present in the human large gut at levels reaching 4 mM [4]. Moreover, IP6 has been detected in plasma, urine and other biological fluids [5]. In terms of human health, dietary IP6 may have both negative and positive roles. It can form tight insoluble complexes with a variety of polyvalent nutritionally important mineral cations, e.g. calcium, iron and zinc, thereby interfering

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with their absorption [6]. This strong chelating capacity of IP6 associated with its six reactive phosphate groups has in the past been regarded as responsible for antinutritional side effects of IP6. However, more recent studies have contradicted those opinions by stating that in evaluation of antinutritional effects of IP6 various factors should be considered, such as the ratio of IP6 to overall mineral content of the diet [7]. It has been shown that the antinutritional effect of IP6 could be manifested only when large amounts of IP6 were consumed together with a diet poor in trace elements, but if essential minerals were present in the proper ratio with respect to IP6, there was no modification of mineral balance [7]. Studies in rats fed IP6 showed no significant toxic effects on serum or bone mineral deficiency [8]. Furthermore, the analysis of IP6 effect on mineral status in rats fed for a long time period through a second generation to evaluate possible effects related to a pregnancy and lactation revealed no decrease in mineral bioavailability, with the exception of lower zinc levels in bone [9]. In addition, rats fed an equilibrated purified diet with IP6 showed about 10-fold higher concentrations of zinc in bone compared to the control animal group [6]. It has also been reported that a high IP6-containing diet did not negatively affect rat plasma copper and zinc concentrations [10] and no relation of zinc deficiency with IP6 has been observed in women who ingested vegetarian or meat-based diet with equal IP6 contents [11].

Over the past few years interest in IP6 has stemmed mostly from its potentially important antineoplastic activity against various types of cancer, including colon cancer [7]. The effectiveness of IP6 as both a cancer preventive and theurapeutic agent has been observed in *in vitro* and *in vivo* experiments, but its mechanisms of action are still not completely understood. [12]. Other IP6 beneficial properties are attributed to a potential hypocholesterolemic and serum lipid-reducing effect [13], inhibition of kidney stone formation [14], prevention of pathological calcification [15] and platelet aggregation [16]. This intriguing molecule has also been shown to act on the host immune functions. IP6 augments NK cell activity *in vitro* and normalizes the carcinogen-induced depression of NK cell activity *in vivo* [17, 18]. It has been shown to act as neutrophil priming agent and up-regulate a number of diverse neutrophil functions [19, 20]. Our previous studies revealed the ability of IP6 to modulate IL-8 and IL-6 secretion from unstimulated intestinal epithelial cells and in response to their stimulation with bacterial endotoxins and IL-1β, which indicates that IP6 present in the intestinal environment may exert immunoregulatory effects on intestinal epithelium [21].

Bacteria of *D. desulfuricans* species are anaerobic, Gram-negative, sulphate-reducing rods, commonly found in the natural environment [22]. The favourable sites for their growth are the anaerobic regions of soil, muds, marine and estuarine sediments. They have been isolated from sea water, saline ponds and sewage, and various polluted environments. These bacteria can utilize sulphate as a terminal electron acceptor in anaerobic respiration, coupling oxidative phosphorylation with reduction of sulphate to produce hydrogen sulphide [23]. Sulphate-reducing *Desulfovibrio* spp. are involved in the degradation of organic matter and play a role in the removal of heavy metals from various environments [24]. On the other hand, these bacteria are responsible for corrosion processes, especially in pumping equipment, storage tanks and pipelines used in oil technologies [25].

Desulfovibrio spp. are also constituents of the normal anaerobic floras of human and animal tracts [26]. However, occasionally they can become opportunistic pathogens [27]. *D. desulfuricans* species have been isolated from appendiceal tissue as well as from patients with acute perforating appendicitis [28]. Cases of gastrointestinal disorders [29], bacteremia [30], and liver abscess [31] caused by these microorganisms have also been described. This can suggest a possible role of certain *D. desulfuricans* components in the pathology associated with infections mediated by these bacteria. Lipopolysaccharide (LPS), the major component of the outer membranes of the walls of Gram-negative bacteria, plays an important role in the pathogenesis and manifestation of Gram-negative infections [32]. After lysis of bacterial cells, LPS is released into circulation and is able to activate the immunological system by stimulating monocytes, macrophages, neutrophils and endothelial cells. The activation of immune cells by LPS leads to a release of inflammatory mediators, i.e. cytokines, chemokines, and enzymes that are responsible for progression of inflammatory reactions and may induce pathophysiological processes, including septic shock [33]. Our previous study on the activity of *D. desulfuricans* endotoxins revealed that they increased the production of cytokines IL-6 and IL-8 in colonic epithelial cells [21, 34]. These endotoxins also stimulated IL-8 and IL-6 secretion in endothelial cells and up-regulated their expression of surface adhesion molecules VCAM1 and E-selectin [35]. Furthermore, endotoxins isolated from *D. desulfuricans* demonstrated greater biological potency in inducing TNF-α secretion from human mononuclear cells at relatively high doses compared to endotoxins from *E. coli* and *S. minnesota* [36].

The purpose of the present study was to evaluate the effect of IP6 on TNF-α secretion by mononuclear cells of peripheral blood in response to their stimulation with LPS from *D. desulfuricans* intestinal and soil strain in comparison to the effects of these cells stimulation with LPS derived from *S. minnesota* Re595 and *E. coli* 0111:B4. The rationale for such a study comes from the fact of possible translocation of microbial flora including *D. desulfuricans* into the blood, due to the increased permeability of the intestinal mucosa associated with some intestinal disorders or medical and surgical treatments. Yet, the local release of IP6 from cells subjected physiological turnover and from damaged or lysed cells *in vivo* can also influence its concentration in extracellular fluids.

Materials and Methods

Bacterial Strains and Isolation of Bacterial Endotoxins

The wild strain DV-A/94 of *D. desulfuricans* was isolated from feces derived from patients suffering from asiderotic anaemia and cholestasis by the method described previously [37]. The *D. desulfuricans* soil strain La 2226 (DMS642) was obtained from the Swiss National Collection of Type Cultures, Lausanne, Switzerland. Bacteria of both strains were cultured anaerobically in Postgate's liquid medium B containing lactate and sulphate [23]. LPS complexes were isolated from bacterial cells according to the hot phenol-water method of Westphal et al. [38]. LPS had no detectable protein, either by silver staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gels or by the Coomassie blue binding assay. Endotoxic activity of isolated LPSs was confirmed by the *Limulus* amebocyte lysate assay according to the manufacturer's instructions, as described previously [34]. In the experiment, LPSs from *S. minnesota* Re595 and *E. coli* 0111:B4 were used as the reference endotoxins. They were purchased from Sigma.

Preparation of LPS and IP6 Concentrations

Stock solutions of 1 mg/ml of LPSs were made in RPMI 1640 culture medium (Sigma) and vortexed for 10 min using ultrasonic bath (Decon Ultrasonic Ltd., England). The dilution of 10 ng/ml of each LPS stock solution in RPMI 1640 medium was used in the study. IP6, as dodecasodium salt from corn, was purchased from Sigma. Stock solution of 100 mM IP6 was prepared in deionized water, pH was adjusted to 7.4 and sterilization was made by filtration. This stock was then diluted to the desired final concentrations (50, 100, 250, 500, 1000 μM) in RPMI 1640 medium.

Isolation of Human Mononuclear Cells

Peripheral blood mononuclear cells were isolated from heparinized peripheral venous blood obtained from consenting healthy volunteers. Blood was diluted 1:1 with phosphate-buffered saline (PBS), pH 7.0, and cell isolation was performed according to a modified Boyüm [39] procedure based on Gradisol L density centrifugation gradient (density, 1.077 g/ml, Polfa, Poland). Gradisol L was stratified on a whole diluted blood which was then centrifuged for 15 min at 600×g at 4°C. After centrifuging, the middle layer containing the mononuclear fraction was taken over to another tube and washed with PBS three times and with RPMI 1640 medium once. Then, cells were suspended in 1 ml of fresh medium and counted under a light microscope using direct Bürker method with the application of Türk liquid chemical (lysis of erythrocytes). The remaining erythrocytes were lysed for 45 s with distilled water, after which concentrated PBS was added to reestablish the isotonic condition. Cells were washed and resuspended in RPMI medium at 10^7 cells/ml and tested for viability and purity. The viability of the cells, judged by erythrosine exclusion, exceeded 95%. The purity of the cell fraction was checked by microscopy of cytospin slides and was greater than 90% for the monocyte fraction.

Stimulation of TNF-α Secretion and Its Immunoassay

Mononuclear cells were suspended at $1 \cdot 10^6$/ml in RPMI medium containing heat-inactivated fetal bovine serum (BIOCHROM KG, Seromed: tested for mycoplasma), 50 μl of β-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma). The resulting cell suspensions were then distributed in 990 μl aliquots in Nunc tissue culture flasks. The 10 μl aliquots of media alone or supplemented with IP6 (50, 100, 250, 500, 1000 μM) were added into the cultures for 5 min, followed by the addition of LPS. Incubation was then continued for 24 h in humidified atmosphere containing 5% CO_2 at 37°C. To determine TNF-α concentration, cell-free supernatants were collected, centrifuged at 800×g for 10 min and frozen at –20°C until assayed. Cytokine concentration was measured using commercially available Quantikine ELISA kit (R&D Systems). The lowest limit of sensitivity for TNF-α was 4.4 pg/ml. Colorimetric results were read on an Elx800 (Biotek Instruments, Inc.) 96-well plate reader at a wavelength of 450 nm. The content of TNF-α was measured within each supernatant in triplicate.

Statistical Analysis

The data were analyzed using one-way ANOVA (p values are presented in Table 1). As a post-hoc analysis, the Tukey HSD test was used and the results of this analysis (p values) are presented in the Results section. All the results are expressed as mean cytokine levels ± SD of the mean of triplicate samples and are representative of three separate experiments, and $p < 0.05$ was considered statistically significant. Statistical analysis was performed using the Statistica PL V 6.0 computer program.

Results

Table 1 presents the levels of TNF-α in pg/ml, released from mononuclear cells incubated with IP6 alone and from cells stimulated with *D. desulfuricans* intestinal and soil strain LPSs and with LPSs from *S. minnesota* and *E. coli* in the absence and presence of IP6. The percentage changes of TNF-α secretion are graphically shown in Figs. 1 and 2. Analysis of TNF-α secretion levels in the cell cultures treated with different concentrations of IP6

Table 1. The levels of TNF-α (pg/ml) released by human mononuclear cells stimulated with LPS (10 ng/ml) from various bacteria in the absence and presence of inositol hexaphosphate (IP6), and by cells incubated with IP6 alone.

IP6 concentration [μM]	No LPS added	LPS from			
		Desulfovibrio desulfuricans intestinal strain	*Desulfovibrio desulfuricans* soil strain	*Salmonella minnesota* Re595	*Escherichia coli* 0111:B4
0	213.8±12.6	975.1±15.3	816.7±5.1	445.0± 5.3	761.6±11.5
50	179.4± 9.1	864.1± 8.8	716.3±2.2	423.5± 4.0	678.8±13.3
100	210.2± 8.5	832.4± 5.0	705.5±6.2	430.2± 5.6	655.2± 7.5
250	250.7±18.1	1009.8±11.6	897.7±7.1	884.3± 8.4	895.4±14.1
500	352.1±10.6	1129.0± 9.3	908.2±7.9	1088.5±15.1	945.3±12.4
1000	571.0±12.2	1129.8± 8.7	983.0±3.7	1105.0± 8.7	1076.7±17.5
One-way ANOVA p	0.004	0.003	0.001	0.0002	0.002

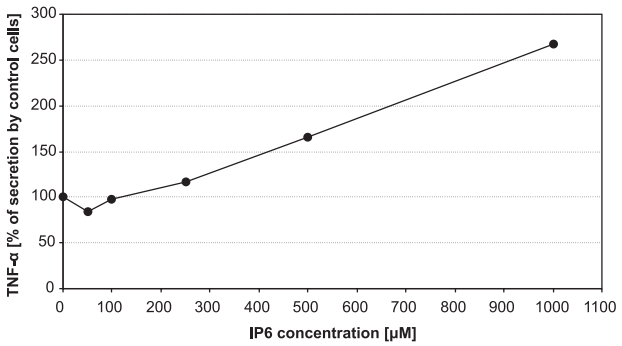

Fig. 1. Profile of TNF-α secretion by human mononuclear cells treated with IP6.

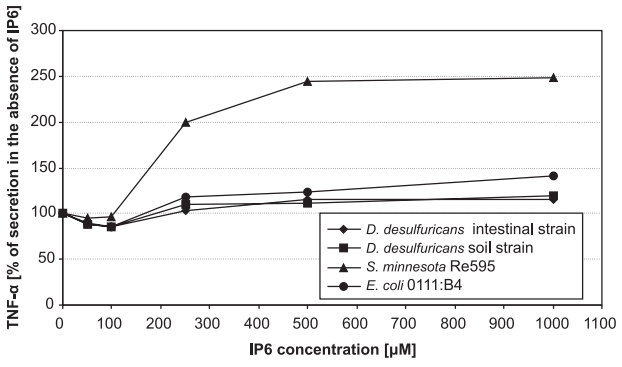

Fig. 2. Profiles of TNF-α secretion by human mononuclear cells stimulated with LPS from various bacteria in the presence of IP6.

alone and with IP6 followed by LPS stimulation revealed significant differences for all investigated cell cultures (one way ANOVA, Table 1). Mononuclear cells showed a constitutive TNF-α secretion of 213.8 ± 12.6 pg/ml in media alone. The level of TNF-α secretion was not affected by the addition of IP6 at the concentrations of 50, 100 and 250 μM because the considered differences in the cytokine amounts released from cells in relation to control cell cultures were statistically insignificant ($p = 0.825$-0.989, Tukey test). An increase in TNF-α secretion occurred upon exposure of cells to IP6 at two higher doses used (500 and 1000 μM). The concentration values of TNF-α released from these cell cultures were significantly above the basal cytokine release ($p = 0.018$ and $p = 0.007$, respectively, Tukey test). As seen in Fig. 1, mononuclear cells treated with 1000 μM IP6 produced higher by 218% and 167% levels of TNF-α than did cells treated with 50 μM IP6 and control cells, respectively.

Incubation of cells with LPS from *D. desulfuricans, S. minnesota* and *E. coli* resulted in a marked release of TNF-α compared to unstimulated control cultures, as indicated by statistical significance, as follows: for LPS from *D. desulfuricans* intestinal strain, $p = 0.003$; for LPS from *D. desulfuricans* soil strain, $p = 0.039$; for LPS from *S. minnesota*, $p = 0.018$; and for LPS from *E. coli*, $p = 0.002$ (Tukey test). Endotoxins from the studied bacterial species differed in biological potency, as reflected by on average two-fold higher levels of TNF-α released in response to LPS from both strains of *D. desulfuricans* compared to the cytokine level secreted following stimulation with *S. minnesota* LPS. The amount of TNF-α released in response to *E. coli* LPS was intermediate between that produced during exposure of cells to LPS from two other bacterial species (Table 1).

Treatment of cells with 50 μM IP6 with subsequent addition of 10 ng/ml stimulating LPS from all bacterial species caused a decrease in secretion of TNF-α as compared to the treatment with LPSs alone, but in case of *S. minnesota* LPS the considered difference was statistically insignificant. Interaction of all endotoxins with cells preincubated with 100 μM IP6 induced marginal changes in TNF-α production when related to the effects observed in

the presence of 50 μM IP6. The treatment with 250 μM IP6 prior to stimulation with bacterial endotoxins revealed differentiation in the level of cytokine secretion. In the case of *S. minnesota* LPS, this effect could be ascribed to the priming of mononuclear cells by IP6, which led to amplification of their response to this endotoxin by 100% in relation to stimulation with LPS alone ($p = 0.001$, Tukey test; Table 1, Fig. 2). This response of the cells exceeded that resulting from the summarized action of S. *minnesota* LPS alone and IP6 alone (Table 1). A more marked enhancement of TNF-α release occurred after incubation of cells with this LPS following 500 μM IP6 ($p = 0.004$, Tukey test), and this effect could not be altered in the presence of 1000 μM IP6 (Table 1, Fig. 2).

Furthermore, in spite of higher amounts of TNF-α released from cells pretreated with 250 μM IP6 and then exposed to LPS from *D. desulfuricans* (both strains) and *E. coli* LPS compared to TNF-α levels evoked by the action of these LPSs on the cells in the absence of IP6, the TNF-α concentration values were statistically significantly ($p = 0.0002$-0.0009, Tukey test) lower than could have resulted from the summarized stimulatory effects of the corresponding LPSs and IP6 alone. This points to the ability of IP6 to diminish the TNF-α secretion induced by these LPSs. Higher doses of IP6 (500 and 1000 μM), though acting as agonists themselves, also appeared to down-regulate the response of cells to LPSs from *D. desulfuricans* and *E. coli*, because the amounts of TNF-α released by cells were below the cytokine levels secreted following the challenges to LPSs alone, plus IP6 alone (Table 1). Doubling the IP6 amount from 500 to 1000 μM did not however, change its influence on the secretion level of TNF-α evoked by *D. desulfuricans* and *E. coli* endotoxins ($p = 0.156$-0.819, Tukey test).

Discussion

Under normal conditions, the gastrointestinal immune system maintains a balance between the immune response to microbial pathogens and tolerance to dietary antigens and local microflora. However, in the inflammatory states of the large gut, abnormal immune response may be directed against enteric bacterial antigens, producing severe inflammatory status of the intestinal mucosa and cellular damage [40]. The origin of LPS in circulation is to an altered permeability of the intestinal barrier and circulating endotoxins have been found in plasma from patients with ulcerative colitis and Crohn's disease [41]. In inflammatory bowel diseases, proinflammatory cytokines have been detected in elevated amounts not only in mucosal tissue, but also in peripheral blood, which suggests monocyte/macrophage stimulation by enteric bacteria and/or their constituents, such as LPS [42].

LPS is a powerful activator of host mononuclear cells and prompts the synthesis and release of multiple cytokines. The production of TNF-α is central in the regulation of the acute inflammatory response to bacterial challenge [43]. TNF-α stimulates endothelial cells to express cell surface adhesion molecules and initiates neutrophil migration. Accumulation of neutrophils and mononuclear cells from the circulation into the gut wall is an integral component of the inflammatory process in the chronic inflammatory bowel diseases. The proportion of mononuclear cells that secrete TNF-α in the lamina propria in patients with inflammatory bowel disease appeared to be increased in an *in vitro* assay [44]. Lipopolysaccharide endotoxins used in this study had different capacities for TNF-α induction from mononuclear cells and LPS from *D. desulfuricans* strains promoted the highest release of this cytokine compared to LPS derived from two other bacterial species. The observed differences in LPS abilities to induce TNF-α could result from structural differences of these complexes. The literature data demonstrated that the LPS polysaccharide chain and phosphoryl group might contribute to the effects of LPS on macrophages [45]. Partial structures of LPS have been reported to reduce cytokine production from mononuclear cells induced by peptidoglycan and LPS [46]. Re595 mutant *S. minnesota* LPS lacks O antigen and outer core polysaccharide [45] and this could account for its lowest capacity for the TNF-α induction from mononuclear cells.

It is thought that anti-carcinogenic benefits of IP6 may in part be attributable to its anti-oxidant capability through its iron chelating properties and, therefore, it has been suggested that it acts as an anti-inflammatory modulator [47]. Intravenously administered IP6 in mice inhibited accumulation of immune effector cells in experimentally induced inflammation [17]. In terms of possible anticancer activity, it has also been suggested that IP6 mediates this action via NK cells because it augmented NK activity in mice with carcinogen-induced colon tumors, and NK activity correlated with tumor suppression [13]. Eggleton et al. [19] reported that the preincubation of human neutrophils with IP6 resulted in substantially enhanced production of reactive oxygen intermediates when the cells were subsequently stimulated by either N-formyl-Met-Leu-Phen (fMLP), phorbol myristate acetate or zymosan. Studies by Crawford et al. [48] revealed the changes in assembly and distribution of F-actin in neutrophils during IP6 priming, with or without fMLP stimulation, which occurred in the absence of any other demonstrable functional responses.

The present study showed that IP6 alone at cellular concentrations (up to 100 μM) and even at 2.5-fold higher dose (250 μM) did not activate mononuclear cells in terms of TNF-α release. By comparison, as reported by Eggleton et al. (19), incubation with IP6 at concentrations up to 250 μM had no effect on neutrophil respiratory burst. This study also showed that at doses significantly exceeding IP6 cellular contents, it acted as an agonist up-regulating TNF-α secretion. Moreover, IP6 appeared to influence differentially the responsiveness of mononuclear cells to secondary stimulus. Up-regulation by IP6 of TNF-α release, referred to as priming, was observed under the influence of *S. minnesota* LPS. Priming is considered one of the regulatory mechanisms implicated in controlling immune cell re-

sponses. This event improves the ability of immune cells to locate and kill invading microorganisms and hence may be critical to effective neutrophil functions [49]. In this connection, IP6 can take part in the defense against invasive bacteria, such as *S minnesota* species. On the other hand, priming is implicated in neutrophil- and lymphocyte-mediated tissue injury both *in vitro* and *in vivo* [49]. In this context, IP6 by down-regulating TNFα release from cells stimulated with *D. desulfuricans* and *E. coli* LPSs which showed higher potency compared to *S. minnesota* LPS in this study, may diminish the tissue damage caused by lymphocytes. Thus, the effects of IP6 released from necrotic cells at an inflammatory focus may be beneficial for a variety of inflammatory diseases, including septic shock.

Recently published studies have indicated immunomodulatory role of IP6 in experimentally-induced endotoxemia in mice [50]. The synergistic administration of IP6 with *Aeromonas hydrophila* endotoxin resulted in an elevation of phagocytic activity compared with the control animal group but still less than that of the LPS given group. Moreover, humoral response represented by changes in the level of the total IgG and IgM antibodies, showed that LPS induced a highly significant elevation in both antibodies, while the IP6 co-administration reduced IgG level. Additionally, marked regenerative effects on the liver histopathological features and ameliorative effects upon the dramatic decrease in total serum protein and albumin profile could be observed in LPS-IP6 treated mice, as compared to LPS treated group.

In conclusion, the present findings demonstrate an extracellular role for IP6, which in this study has appeared to act as a bi-functional modulator of TNF-α release from mononuclear cells in response to bacterial challenge. The enhancing or diminishing effects of IP6 may control the level of activation states and subsequent responses of mononuclear cells, depending on the particular Gram-negative bacteria' endotoxins.

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