Evaluation of Biochemical Differences and Immunological Effects of LPS and Lipid a Extracted from Brucella Strains

Mariam Khatami 1, Zahra Kianmehr2, Maliheh Safavi3, Sussan Kaboudanian Ardestani4*, Nasser Ghaemi5

1. Production and Research Complex, Pasteur Institute of Iran, Karaj, Iran
2. Faculty of Biological Sciences, Islamic Azad University, North Tehran Branch, Tehran, I.R Iran
3. Department of Biotechnology, Iranian Research Organization for Science and Technology, Tehran, Iran
4. Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
5. School of Chemistry, College of Science, University of Tehran, Tehran, Iran

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Abstract

Background: The intrinsic heterogeneity determination in Brucella Lipopolysaccharide (LPS) is important for explaining its chemical nature and biological behavior. This is significant for practical purposes, since LPS is the most relevant antigen during infection and vaccination. The purpose of the present study was to compare biochemical and immunological differences of LPS and lipid A in three strains of Brucella: B. melitensis (virulent strain), B. melitensis (vaccine strain, Rev1), and B. abortus (vaccine strain, S19).

Materials and Methods: LPSs were extracted from Brucella strains using hot phenol-water method, and lipid A was obtained through mild acid hydrolysis. Glycan, phosphate, KDO, and protein concentration were evaluated in both LPS and lipid A samples. Immunological effects of Brucella LPS and lipid A were investigated measuring mitogenesis, IL-6, and Nitric Oxide (NO) production.

Results: LPS and lipid A of B. melitensis have more glycan, KDO, protein, and phosphate compared with B. abortus. Different species of Brucella LPS and lipid A induced NO production in a time- and dose-dependent manner via J774A.1 cells. One μg/ml LPS extracted from different strains of Brucella can induce maximum NO production. However, lipid A from S19 cannot induce NO and lipid A from B. melitensis induces NO production in higher doses of KDO than its LPS. Maximal production of IL-6 and higher mitogenic index in human lymphocytes was observed by Rev1 LPS.

Conclusions: Regarding the diverse biochemical and immunostimulatory properties of LPS and lipid A, these strains of Brucella can be used potentially for different approaches, such as designing subunit brucellosis vaccines or effective adjuvants. For instance, LPS from B. abortus, as an effective and safe adjuvant due to its less toxicity, and Rev1 LPS, as subunit vaccines in developing anti-Brucella vaccines due to its high immunopotency, have been applied in several studies.

Keywords: Brucella strains; LPS; Lipid A; IL-6; NO production; Mitogenic index

Introduction

Brucella spp are Gram-negative facultative intracellular pathogens that infect humans and domestic animals (1, 2). The genus Brucella, according to antigenic variation and host preference, consists of seven species: Brucella melitensis, B. suis, B. abortus, B. ovis, B. canis, B. neotomae, and B. maris (2, 3). Two of the main pathogenic species, which bear the antigen characteristic of smooth (S)-type brucellae, are B. abortus, responsible for bovine brucellosis, and B. melitensis, the main etiologic agent of ovine and caprine brucellosis. Brucellosis is a disease
that causes abortion in ewes and goats resulting in huge economic losses, particularly in Mediterranean countries (4). Furthermore, these pathogens cause brucellosis in humans, leading to chronic stages of the disease that can be manifested as orchitis, spondylitis, arthritis, and a debilitating illness known as undulant fever (5). The most important virulence factor of the infection by Brucella is lipopolysaccharide (LPS) so that LPS deficient strains have less virulence and intra-cellular survival potency (6). LPS is an amphipathic molecule with three covalently linked regions: O-specific polysaccharide, core oligosaccharide, and lipid A (7, 8). LPS interacts with membrane receptors on target cells such as Toll-like receptor 4 (TLR4) thereby initiating a cascade of signal transduction events. The end result of the intracellular signaling is mainly the activation of NF-κB, which then induces the expression of diverse genes such as proinflammatory cytokines including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-12 and adhesion molecules, etc (9, 10).

Nitric Oxide (NO) is a free radical that plays an important role in several biological processes such as macrophage-mediated cytotoxic activity against a variety of pathogens including bacteria, viruses, and parasites (11). NO formation is catalysed by three different isoforms of Nitric Oxide Synthase (NOS) that convert L-arginine into NO and L-citrulline (12). Expression of the inducible isoform of NOS (iNOS) in activated macrophages is mainly responsible for production of pathological concentration of NO during inflammation. iNOS expression is stimulated by proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1 as well as by microbial LPS (13, 14), so that LPS and lipid A of B. abortus and B. melitensis could induce the production of NO by rat adherent peritoneal cells (15).

It is significant to determine the intrinsic heterogeneity in Brucella LPS so that we can explain its chemical nature and biological behavior. Because LPS is the most relevant antigen during infection and vaccination, this analysis is crucial from practical perspectives. In addition, LPS and LPS-related molecules are widely implemented in immunological investigations as well as the diagnosis of brucellosis (4, 16, 17).

In the present study, we investigated biochemical differences including the required time for completion of hydrolysis of LPS and subsequent lipid A isolation, quantity of KDO (2-keto-3-deoxyoctonic acid), glycan, phosphate, protein content in purified LPS, and isolated lipid A from three strains of Brucella. Next, the biological activities of these components, such as capability of NO production in murine macrophage cell line (J774A.1), IL-6 production, and mitogenesis index in human lymphocytes treated with LPS. Since most biological effects of LPS have been associated with the lipid A moiety (18), we also investigated these properties by Brucella lipid A and compared these effects with those of Escherichia coli (E.coli) LPS.

Materials and Methods

Bacterial strain

Three strains of Brucella, including B. melitensis (virulent strain), B. melitensis (vaccine strain, Rev1), and B. abortus (vaccine strain, S19), were obtained from the type bacteria collection of Razi Institute of Iran (Tehran, Iran).

LPS extraction

Extraction of LPS was performed using hot-phenol method, according to Leong method (19). In brief, 1.5 g wet weight of cells was suspended in distilled water, followed by sonication for 90s. The lyzed cells were added to phenol solution (90% w/v) and maintained at 67ºC for 15 min. The mixture was centrifuged for 30 min in 6000 g and phenol layer was removed. The LPS was precipitated from phenol layer using three volumes of cold methanol solution (99% methanol, 1% sodium acetate) and resuspended in distilled water. For further enzymatic digestion, DNase (10 unit/ml), RNase (10 unit/ml), lysozyme (25 µg/ml), and Proteinase K (100 µg/ml) were added to reduce nucleic acid and protein contamination of the extracted LPS samples. Finally, LPS solution was dialyzed against distilled water, lyophilized, and then stored at-20ºC until use.

Chemical analysis of extracted LPS

The thiobarbituric method was used to measure KDO as a unique component of LPS and the most popular marker of LPS from gram-negative bacteria (20). Glycan test and phosphate assay were carried out according to Raff and Wheat (21) and Ames (22), respectively. Bradford method was used for measuring protein content of the samples. The quality of extracted LPS was determined...
using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. LPS-specific silver staining was performed according to Tsai and Frasch (23). Specific assessment of lipids and carbohydrates was performed using sudan black (24) and periodic acid Schiff staining (25), respectively. LPS of Salmonella enteritidis was used as standard in SDS-PAGE.

**Isolation of lipid A from Brucella LPS**

Lipid A was obtained from three Brucella strains via hydrolysis of purified LPSs with 2% acetic acid at 100°C. In order to determine the suitable time for completing hydrolysis of LPS and isolation of lipid A, acidic hydrolysates were performed in different time courses of one to six hours. Hydrolysis product included pellet and supernatant that contained lipid A and oligosaccharide segments, respectively. Supernatant was discarded and the lipid A in pellet samples were collected, freeze dried, and stored at -20°C until use.

**Evaluation of NO production in J774A.1 cells stimulated with LPS and lipid A Brucella strains**

Murine macrophage cell line (J774A.1) was purchased from National Cell Bank (Pasteur Institute of Iran) and cultured at 37°C in DMEM medium (GIBCO, Invitrogen, Germany), supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Invitrogen, Germany), 0.584 g/l L-glutamine, sodium bicarbonate 3.7 g/l, 50 µg/ml penicillin, and 50 µg/ml streptomycin. Adherent J774A.1 monolayers were obtained plating the cells in 24-well tissue culture plates at 1-106 cells/well for 18 h at 37°C. The cells were stimulated with different concentrations (0.01, 0.02, 0.2, 0.4, 1, 2, 10 and 20 µg/ml) of LPS and lipid A isolated from three strains of Brucella. Different concentrations of LPS and lipid A were prepared based on KDO content. In order to determine NO production by macrophage, NO-2 and NO-3 were measured in cell free culture supernatant of wells after 24 h and 48 h incubation at 37°C using griess assay (26).

For NO-2 assay, the culture medium (100 µl) was mixed with 100 µl of griess reagent, and after 10 minutes incubation at room temperature, the absorbance was measured at 540 nm using an ELISA reader (Gen 5, Biotek, US). For NO-3 assay, the culture medium (100 µl) was mixed with 100 µl vanadium chlorides, which leads to reduction of NO-3 to c, and 50 µL of griess reagent. The mixture was incubated at room temperature for 40 min and absorbance was measured at 540 nm. E. coli LPS (0.1 µg/ml) was used for comparison with B. strains LPS induction. Un-stimulated J774 cells, receiving an equal amount of RPMI the same as LPS and lipid A under similar culture conditions, were considered as negative control. The PHA (Merck) (70 µg/ml) was used in each assay as positive control.

**Assessment of IL-6 production**

Lymphocytes were isolated from human whole blood of healthy donors via centrifugation in 800 g for 20 min on Ficoll density gradient. After washing three times with RPMI 1640 medium, cells were cultured in RPMI 1640 medium (GIBCO, Invitrogen, Germany), supplemented with antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin) and 10% FBS in 96-well culture plates at 37°C to a density of 2.6-104 cells/well. Cultured lymphocytes were stimulated with different

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**Table 1: Characterization of Purified LPS and Lipid A from Brucella strains**

<table>
<thead>
<tr>
<th>Brucella strains</th>
<th>Purified sample</th>
<th>KDO (µg/ml)</th>
<th>Glycan (µg/ml)</th>
<th>Phosphate (µg/ml)</th>
<th>Protein (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. melitensis</em></td>
<td>LPS</td>
<td>30.35</td>
<td>785</td>
<td>20</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>LPS after acidic hydrolysis*</td>
<td>--------</td>
<td>341</td>
<td>22</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>Lipid A**</td>
<td>23</td>
<td>34.14</td>
<td>10.7</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>23.2</td>
<td>618</td>
<td>27</td>
<td>160</td>
</tr>
<tr>
<td><em>B. melitensis</em> (Rev1)</td>
<td>LPS, after acidic hydrolysis</td>
<td>--------</td>
<td>330</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Lipid A</td>
<td>22.5</td>
<td>29</td>
<td>4.8</td>
<td>34.45</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>19</td>
<td>412</td>
<td>22</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>LPS after acidic hydrolysis</td>
<td>--------</td>
<td>300</td>
<td>23</td>
<td>110</td>
</tr>
<tr>
<td><em>B. abortus</em> (S19)</td>
<td>Lipid A</td>
<td>7</td>
<td>10.64</td>
<td>5.7</td>
<td>17.1</td>
</tr>
</tbody>
</table>

* Before centrifuge
** Pellet, after centrifuge
concentrations (0.01-20 µg/ml, in triplicate) of LPS and lipid A isolated from three strains of *Brucella*. The PHA (70 µg/ml) was used as positive control. The levels of the IL-6 production were determined in culture supernatant of each well after 48 h using commercial ELISA kit (R&D system, Minneapolis, MN) according to the manufacture’s instruction.

**Mitogenesis assay of human lymphocytes**

Assessment of mitogenesis was performed using lymphocytes transformation test (LTT). Briefly, isolated lymphocytes from human whole blood were grown in 96-well culture plates at 37ºC in a humidified CO2 incubator to a density of 2.6-10⁴ cells/well and stimulated for proliferation with different concentrations (0.01-20µg/ml, in triplicate) of LPS and lipid A. The PHA and RPMI medium were used in each assay as positive and negative control, respectively. After 48 h of incubation at 37ºC, 20µl (3 h) thymidine (50 µCi/ml) was added to each well and further incubated for 6 h. The results were analyzed using β counter instrument (WALLAC 1410, Switzerland).

**Statistical method**

The values of experiments are expressed as mean±S.D. The significance of differences between samples was tested using Student’s t-test. Values of P<0.05 were considered statistically significant.

**Results**

**LPS characterization**

The quality of the *B*. strains LPS was demonstrated using KDO assay, glycan test, and special silver nitrate staining following electrophoresis on SDS-PAGE. As expected, purified LPS had heterogeneous structure and seemed as a smear with two distinctive band zone (low and high molecular weight) on silver-stained SDS-gel (Figure 1). Suitable time for complete hydrolysis of LPS and isolation of lipid A from *B*. strains was obtained based on assessment of hydrolysis product using electrophoresis on SDS-PAGE. As shown in Figure 2, completely hydrolyzed LPS and isolated lipid A have more migration and also oligosaccharides area was increased on gel. According to the results of SDS-PAGE, the appropriate time for acidic hydrolysis was 5.5 h for *B. melitensis*, 5 h for Rev1, and 3 h for S19 for complete hydrolysis of LPS and release of the entire lipid A.

![Figure 1. Silver stained SDS-PAGE profiles of LPS extracted from the *Brucella* strains. *B. melitensis* (Rev1) (lane 1, 2), *B. melitensis* (virulent strain) (lane 3, 4), *B. abortus* S19 (lane 5, 6) and LPS of Salmonella enteritidis as standard (lane 7). The slower-migrating smear in all lanes represent intact LPS (i.e., Lipid A, core, and 0-linked sugars) and the fast-migrating bands in all lanes represent Lipid A without 0-linked sugars.](image1)

![Figure 2. Silver stained SDS-PAGE profiles for acidic hydrolysis product of LPS from the *Brucella* strains. LPS of *Salmonella enteritidis* as standard (lane 1), LPS of *B. melitensis* (Rev1) (lane 2), LPS of *B. melitensis* (Rev1) after 5 h acidic hydrolysis (lane 3), LPS of *B. abortus* S19 (lane 4), LPS of *B. abortus* S19 after 3 h acidic hydrolysis (lane 5), LPS of *B. melitensis* (lane 6), LPS of *B. melitensis* after 5.5 h acidic hydrolysis (lane 7) and molecular weights marker (lane 8).](image2)
Sudan black and periodic acid Schiff staining were used for characterization of lipid A (Fig. 3). Top panels of the gel showed the electrophoretic separation of carbohydrates bands stained with periodic acid Schiff (Figure 3) and bottom panels shows the presence of carbohydrates stained with Sudan black (Fig. 3B). The results of KDO, glycan, phosphate assay, and Bradford test of samples are presented in Table 1. As shown, LPS and lipid A of *B. melitensis* have higher amounts of KDO (30.35, 23µg/ml), glycan (758, 34.14 µg/ml), phosphate (20, 10.7 µg/ml), and protein (220, 44µg/ml) compared with other two strains. Also, the samples contain some proteins even after purification and acidic hydrolysis of LPS because of tight interaction between several outer membrane proteins and LPS.

**Measurement of NO\textsuperscript{2} and NO\textsuperscript{X} in J774A.1 cells stimulated with LPS and lipid A**

Since *E. coli* LPS is a microbial product inducing NO production by murine macrophages (27), it was used as a control of our cellular system. After 24 h, *B. melitensis* LPS showed significant levels of NO\textsuperscript{2} production from dose of 0.01µg/ml whereas S19 and Rev1 LPS produced NO\textsuperscript{2} production from doses of 0.2 and 0.4µg/ml, respectively. After 48 h, *E. coli* LPS induced NO\textsuperscript{2} production twice its amount in 24 h while as for *Brucella* strains LPS, the obtained results were similar to those for 24 h and there was a significant difference only in 1µg/ml of *Brucella* strains LPS *B. melitensis* and Rev1 LPS (Fig. 4A). Since lipid A has major impacts on the biological effects of the LPS, we also studied the ability of these lipids to induce...
Figure 5. Production of NO$^+$ by murine macrophage cell line (J774A.1)

Murine macrophage cell line stimulated with different concentrations of (A) *B. melitensis*, Rev1 and S19 LPS (0.01, 0.2, 0.4 and 1 µg/ml), (B) *B. melitensis* and Rev1 lipid A (0.01, 0.02, 0.2, 0.4, 1, 2, 10 and 20 µg/ml), (C) S19 LPS (0.4 µg/ml) and Lipid A (0.01, 0.02, 0.2, 0.4, 1 and 2 µg/ml). NO$^+$ production was assayed 24 h and 48 h after stimulation. *E. coli* LPS (0.1 µg/ml) was used for comparison with *Brucella* strains LPS induction and PHA (70 µg/ml) was used as positive control. Data are the means of duplicate sample results. The levels of statistical significance for differences between test groups were determined by the student’s t-test. *P*<0.05, in comparison to control after 24 h and *4P*<0.05, in comparison to control after 48 h.

NO production. As illustrated in Fig. 4B, *Brucella* strains lipid A showed almost similar potencies to elicit NO induction. After 24 h, significant amounts of NO$^+$ were obtained in a majority of *B. melitensis* (virulent strain and Rev1) lipid A concentration and the maximal NO$^+$ production was obtained with concentration of 2 and 10µg/ml lipid A *B. melitensis* and Rev1, respectively. There was no significant increase in NO$^+$ production by S19 lipid A after 24 h in different concentrations (Figure 4C). So, in other test, after incubation of cells with different concentrations of S19 lipid A for 2 h, supernatant was harvested, 0.4µg/ml S19 LPS was added to new medium, and NO$^+$ production was measured after 24 h and 48 h. As shown in Fig 4C, this method, however, did not increase NO$^+$ production. The effects of LPS and lipid A on NO$^+$ production are shown in Fig. 5. Measurement of NO$^+$ in stimulated J774A.1 cells with LPS and lipid A demonstrated that the amount of NO$^+$ is higher than those of NO$^-$ in 24 h and 48 h (Figure 5). In the present study, often, LPS and lipid A from different *Brucella* strains induced NO$^-$ production in a time- and dose- dependent manner; however, LPS + Lipid A from S19 was not observed to have a significant effect on NO$^-$ and NO$^+$ production.

Discussion

IL-6 production and mitogenesis in human cultured lymphocytes

Three *Brucella* strains LPS stimulated cell proliferation and production of IL-6 in a dose-dependent manner. *B. melitensis* and Rev1 LPS showed significantly higher cell proliferation and IL-6 production (*P*<0.05) than S19 LPS (Figure 6,7). There was no significant difference in IL-6 production levels induced by three *Brucella* strains lipid A. IL-6 production levels induced by lipid A were much lower than that of LPS in all strains (Fig 6). The impact of lipid A on cell proliferation was similar to that of LPS but lipid A inhibited mitogenesis at 8µg/ml dose in *B. melitensis* and S19 strains (Figure 7).

The chemical composition and most of the biological activities induced by *Brucella* LPS considerably differ

Figure 6. Measurement of IL-6 production in human cultured Lymphocytes stimulated with different concentrations of *Brucella* strains LPS and lipid A (0.04, 0.4, 4 and 8 µg/ml) *B. melitensis* and Rev1 LPS showed higher IL-6 production than S19 LPS.
Figure 7. Measurement of mitogenesis in human cultured lymphocytes stimulated with different concentrations of Brucella strains LPS and lipid A (0.04, 0.4, 4 and 8 µg/ml). B. melitensis and Rev1 LPS showed higher mitogenic index than S19 LPS. *Significance difference in Comparison to control (P< 0.05).

from those of enterobacterial LPS such as E. coli (7), for instance Brucella LPS is nonpyrogenic and less toxic (28). The differences in toxicity between Brucella and E. coli LPS may be related to the variations in their lipid A structures. Brucella lipid A significantly includes large proportion of long-chain saturated and unsaturated fatty acids (>C16), very small amounts of hydroxylated fatty acids, no β-OH myristic acid, and a proportion of amide- and ester-linked fatty acids, which is different from that in enterobacteria, such as E. coli. Other differences are found in the composition of the core polysaccharide and O-antigen side chain of the complete LPS molecule (29, 30).

Indeed, Brucella LPS are a family of related molecules which display heterogeneity not only at the level of the O-polysaccharide, but also at the core oligosaccharide and the lipid A (16). The degree of heterogeneity in Brucella lipid A may depend mainly on the various fatty acid substitutions. The lack of backbone constituents (other than lipids) and ester-linked acyl-oxacyl residues in Brucella lipid A might account for the limited number of variants as compared to enterobacterial lipid A (16). In the current study, we compared biochemical differences and immunological effects of LPS and lipid A moieties in three strains of Brucella (B. melitensis, Rev1 and S19). Our results showed that S19 has lower glycan, KDO, protein content, and phosphate than the other Brucella strains. High amount of KDO in B. melitensis is consistent with the previous observations (15). Suitable time for complete hydrolysis of LPS and isolation of lipid A according to SDS-PAGE pattern in Brucella strains (3-5 h) was more than that reported for other bacteria such as E. coli (1-2 h). In fact, the bacteria with high amounts of glycan required more time for complete hydrolysis of LPS. The data showed that B. melitensis LPS has higher glycan amount (758 µg/ml) and subsequently required more time for complete hydrolysis of LPS (5.5 h) in comparison with Rev1 (618 µg/ml, 5 h) and S19 (412 µg/ml, 3 h). It was also found that lipid A extracted from LPS Brucella strains has little amounts of phosphate; this result is in agreement with those reported by Freer et al and i. Moriyon et al. (16, 31).

Measurement of NO production in stimulated J774A.1 cells with LPS and lipid A indicated that different Brucella strains of LPSs and lipid As generated NO in a time- and dose-dependent manner. Our results revealed that LPS and lipid A from B melitensis induce NO production more than the other two strains. Brucella strains LPS showed significant levels of NO production after 48 h, whereas there was no significant increase in the NO production by Brucella strains lipid A after the same time. Since different concentrations of S19 lipid A could not significantly increase NO₂⁻ production, we used S19 LPS (0.4 µg/ml) as stimulator; however, it did not prove to be an effective approach. It seems that particular receptors on cells were occupied by lipid A. Comparison of stimulatory effect of S19 lipid A on NO₂⁻ and NO⁺ production revealed that S19 lipid A results in significant increase in NO⁺, probably because it produces superoxide, in addition to NO₂⁻, both of which can invert into NO⁺. B. melitensis and Rev1 LPS induced higher mitogenic index than S19 LPS, which is in agreement with the data reported by Qureshi et al. (32). Increase in the amounts of Brucella strains LPS and lipid A results in growth stimulation and reduction of mitogenic index, respectively.

Schrommet et al (33) showed that variation in biological activities of LPSs is directly correlated with the molecular shape of their lipid A. Only (hexa acyl) lipid A with a conical/concave shape displayed strong endotoxic activity, i.e. IL-6 inducing capacity. Moreover, cylindrical molecular shape of lipid A is correlated with antagonistic activity, so that IL-6 induction by LPS was inhibited by cylindrically shaped lipid A except for the compounds with reduced head
group charge. The antagonistic action is explained by the assumption that lipid A molecules intercalate into the cytoplasmic membrane of mononuclear cells, and subsequently block the putative signaling protein by the lipid A with cylindrical shape. Therefore, we assessed Brucella strains lipid A ability in IL-6 induction as the pre-inflammatory cytokine. The results of the present study indicated that Rev1 LPS is a stronger stimulator of IL-6 production whereas there was no difference in IL-6 production between lipid A Brucella strains. Based on our results, LPS and lipid A Brucella strains induced different levels of IL-6. Therefore, different immune responses will be expected when these compounds are used as adjuvants in animal models.

In summary, Brucella strains induce different levels of IL-6 production. Therefore, different immune responses will be expected when these compounds are used as adjuvants in animal models. Based on our results, LPS and lipid A Brucella strains induced different levels of IL-6. Therefore, different immune responses will be expected when these compounds are used as adjuvants in animal models. In conclusion, according to the differences in biochemical and immunostimulatory properties of LPS and lipid A, these strains of Brucella can potentially be used for different approaches such as designing subunit vaccines or effective adjuvants.

References

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