Immunization against leishmaniasis by PLGA nanospheres loaded with an experimental autoclaved \textit{Leishmania major} (ALM) and \textit{Quillaja} saponins

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Abstract. Immune responses against the \textit{Leishmania} antigens are not sufficient to protect against a leishmania challenge. Therefore these antigens need to be potentiated by various adjuvants and delivery systems. In this study, Poly (d,l-lactide-co-glycolide (PLGA) nanospheres as antigen delivery system and \textit{Quillaja} saponins (QS) as immunoadjuvant have been used to enhance the immune response against autoclaved \textit{Leishmania major} (ALM). PLGA nanospheres were prepared by a double-emulsion (W/O/W) technique. Particulate characteristics were studied by scanning electron microscopy and particle size analysis. Mean diameter for nanospheres loaded with ALM+QS was 294 ± 106 nm. BALB/c mice were immunized three times in 3-weeks intervals using ALM plus QS loaded nanospheres [(ALM+QS)PLGA], ALM encapsulated with PLGA nanospheres [(ALM)PLGA], (ALM)PLGA+QS, ALM + QS, ALM alone or PBS. The intensity of infection induced by \textit{L. major} challenge was assessed by measuring size of footpad swelling. The strongest protection, showed by significantly (P < 0.05) smaller footpad, were observed in mice immunized with (ALM)PLGA. The (ALM+QS)PLGA group showed the least protection and highest swelling, while the (ALM)PLGA+QS, ALM+QS and ALM showed an intermediate protection with no significant difference. The mice immunized with ALM and ALM+QS showed the highest IgG2a/IgG1 ratio (P<0.01), followed by (ALM)PLGA+QS. The highest IFN-γ and lowest IL-4 production was seen in (ALM)PLGA+QS, ALM+QS groups. The highest parasite burden was observed in (ALM)PLGA+QS and (ALM+QS)PLGA groups. It is concluded that PLGA nanospheres as a vaccine delivery system could increase the protective immune responses, but QS adjuvant has a reverse effect on protective immune responses and the least protective responses were seen in the presence of this adjuvant.

INTRODUCTION

Leishmaniasis depending upon the parasite species and the host immune response, represents a wide spectrum of clinical manifestations, ranging from a self-healing cutaneous lesion to fatal visceral disease. It is estimated that 350 million people are at risk of leishmaniasis, 12 million are affected and the annual incidence is about 2 million (http://www.who.org; Rafati \textit{et al.}, 2007; Palatnik-de-Sousa, 2008).

The main effect of particulate adjuvant/delivery systems is the effective delivery of antigen for enhanced uptake by APCs, and DCs in particular. These adjuvants are most often taken up by classical APCs, such as DCs and macrophages, whereas B cells are not the prime target for these delivery systems (Lycke, 2007). Biodegradable
poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres and microspheres are promising delivery systems for protein, peptide and DNA vaccines (Johansen et al., 2000; Diwan et al., 2002). Nano- and microparticles enhance the immune responses against encapsulated antigen by various mechanisms. They could impart particulate nature to soluble antigens and increase their interaction with antigen presenting cells (APCs)(Sajadi Tabassi et al., 2008). They can deliver peptide antigens to APCs (Newman et al., 2002) to generate Th1 type immune response, even against poor immunogens (Newman et al., 1998; Venkataprasad et al., 1999). They have been used to co-encapsulate the antigen and adjuvant, to deliver both of them to the same APC and result in induction of stronger immune response compared to the free antigen and adjuvant (Diwan et al., 2002).

The immunoadjuvant effect of Quillaja saponins (QS), extracted from Quillaja saponaria bark, is shown in several studies (Huber et al., 2002; Zhang et al., 2003; Ellis et al., 2005). Saponins induces Th1, CD8+ response and, IgG2a/ IgG2b antibody response (Gupta & Siber, 1995). The possible mechanism of immunoadjuvant effects of saponins is by formation of mixed-micelles with cell proteins (Gupta & Siber, 1995; Cox & Coulter, 1997). Controlling the cytokine production, clonal differentiation of complement cells in lymph nodes, enhancing the CTL and natural killer cell activity, mitotic effect on lymphocytes and activation of macrophages and granulocytes have also been attributed to saponins (Gupta & Siber, 1995; Cox & Coulter, 1997; Lacaille-Dubois, 1999; Singh & O’Hagan, 1999).

The adjuvant effect of Quillaja saponins is proposed to be related to a functional aldehyde-containing group present within the individual molecules and aliphatic side-chains (Soltyskik et al., 1995). Quil A has been shown to intercalate into cell membranes through interaction with structurally similar cholesterol, forming ‘holes’ or pores (Glaueri et al., 1962). This may allow antigen to gain access to the endogenous pathway of antigen presentation and promote a CTL response, however it is currently unknown if the adjuvant effect of saponins is related to pore formation (Sjölander et al., 2001; Singh & O’Hagan, 2003).

The immunogenicity of antigen and potency of an adjuvant could be substantially enhanced by co-delivery in particulate delivery systems, like microspheres (Diwan et al., 2002). Therefore, in this study PLGA nanospheres encapsulated with autoclaved leishmania major (ALM) and Quillaja saponin adjuvant were studied for immunization against leishmaniasis.

MATERIALS AND METHODS

Materials

PLGA 50:50 co-polymer (MW 30000) was purchased from Boehringer Ingelheim (Germany). Purified Quillaja saponin was purchased from Sigma (St Louis, USA). Autoclaved Leishmania major (ALM) produced at Razi Vaccine and Serum Research Institute, Hesarak, Iran and used as experimental vaccine in clinical trials (Bahar et al., 1996; Alimohammadian et al., 2002; Kamil et al., 2003; Mohebali et al., 2004; Khamesipour et al., 2006).

Female BALB/c mice 6-8 weeks old were purchased from Pasteur Institute (Tehran, Iran). The mice were fed with tap water and standard laboratory diet (Khorassan Javane Co, Mashhad, Iran). Animals were housed in a colony room 12/12 h light/dark cycle at 21°C and had free access to water and food. Animal experiments were carried out according to Mashhad University of Medical Sciences, Ethical Committee Guidelines.

The L. major strain (MRHO/IR/75/ER) used in this experiment is the same strain which has been used for preparation of experimental Leishmania vaccine and leishmanization (Javadian et al., 1976; Bahar et al., 1996; Alimohammadian et al., 2002; Kamil et al., 2003; Mohebali et al., 2004; Khamesipour et al., 2006; Noazin et al., 2008, 2009).
Soluble *Leishmania* Antigen (SLA) was prepared from promastigotes of *L. major* harvested at log phase, and stored in small aliquots at -70ºC until use, the protein concentration of SLA was determined using Lowry protein assay (Scott *et al*., 1987).

**Preparation and characterization of PLGA nanosphere encapsulated with ALM and QS**

Nanospheres were prepared using a W/O/W emulsion and solvent evaporation technique (Tafaghodi *et al*., 2004). Briefly, QS (40 µl, 10 µg/µl) and ALM (110 µl, 70 µg/µl) solutions were mixed and emulsified with PLGA solution (600 µl, 33% w/v) in dichloromethane for 40 s using a microtip probe sonicator (MSE, UK) in amplitude 18. Ice-water bath was used for prevention of temperature rise in sonication processes. The W/O emulsion was then combined with PVA solution (8 ml, 7.5% w/v) and sonicated (80 s) to form the W/O/W emulsion. The secondary emulsion was then added to PVA solution. The emulsion was further stirred for 2 h. Nanospheres were collected by centrifugation (20,000 g, 15 min, 4ºC) washed twice with distilled water, and then lyophilized.

Scanning electron microscope (Leo, Germany) was used to study the morphology of nanospheres. Particle size and size distribution of the nanospheres were determined using a laser diffraction size analyzer (Shimadzu, Japan). The amount of encapsulated ALM and QS was determined by using Lowry protein assay method (Waterborg, 2002).

**Immunization of BALB/c mice**

Different groups of mice, 10 mice per group, were subcutaneously (SC) immunized 3 times at 3 weeks intervals with one of the following formulations: 1- (ALM+QS)$_{PLGA}$ (180 µg ALM + 10µg QS/10 mg nanosphere/100µl PBS/mouse), 2- (ALM)$_{PLGA}$+QS (180 µg ALM/10 mg nanosphere + 10 µg QS/100µl PBS/mouse), 3- (ALM)$_{PLGA}$ (180 µg ALM /10 mg nanosphere /100 µl PBS /mouse), 4- ALM+QS (180 µg ALM + 10µg QS/100 µl PBS/mouse), 5- ALM (180 µg ALM/100 µl PBS/mouse) 6- PBS (100 µl).

**Challenge with *L. major* promastigotes**

The immunized mice (7 per group) were challenged SC into left footpad with 1.5x10$^6$ *L. major* promastigotes harvested at stationary phase (in 50 µl volume), at 3 weeks after the last booster and as a control right footpads were injected with the same volume of PBS. Size of lesion were recorded in each mouse by measurement of footpad thickness using a metric caliper (Mitutoyo Measuring Instruments, Japan). Grading of lesion size was carried out by subtracting the thickness of uninfected contralateral footpad from that of the infected one (Jaafari *et al*., 2006, 2007).

**Antibody isotype assay**

Blood samples were collected from the mice before and at week 14 after challenge and the sera were used to titrate anti-*Leishmania* IgG total, IgG1 and IgG2a antibodies using ELISA method (Zymed Laboratories Inc., San Francisco, USA) according to the manufacturer’s instructions. Briefly, 96-well microtiter plates (Nunc) were coated with 50 µl of 10 µg/ml of SLA overnight at 4ºC. Plates were washed and blocked with 1% bovine serum albumin in Tween (PBS-Tween). Serum samples were diluted to 1:200 with PBS–Tween and 50 µl of diluted serum was added to each well. Optical density was determined at 450 nm using 630 nm as the reference wavelength (Badiee *et al*., 2007).

**In vitro spleen cell responses**

Three mice from each group were sacrificed at week 3 after the last booster, at the same time as challenge experiment, the spleens were aseptically removed and a single-cell suspension was prepared by homogenization of the tissue, and the erythrocytes were disrupted using ammonium chloride. The splenocytes were washed and re-suspended in complete medium (RPMI 1640-FCS) and seeded at 2x10$^6$/ml in 96-well flat-bottom plates (Nunc). The spleen cells were stimulated *in vitro* with SLA (10 µg/ml) or Con A (2.5
µg/ml), or medium alone and incubated at 37°C with 5% CO2. Supernatants were collected at 72 h of culture and the concentration of IL-4 and IFN-γ were titrated using the ELISA method according to the manufacturer's instructions (Bender Med Systems GmbH, Vienna, Austria) (Badiee et al., 2007).

Statistical Analysis
One-way ANOVA statistical test was used to assess the significance of the differences between various groups. In case of significant F-value multiple comparison Tukey test was used to compare the means of different treatment groups, P < 0.05 was considered to be statistically significant.

RESULTS

Characterization of PLGA nanospheres
As shown in Figure 1, spherical nanospheres with a smooth surface were prepared. Encapsulates did not affect the morphology and surface roughness of nanospheres. The yield percentage of nanospheres encapsulated with ALM or both ALM and QS was determined as 49.4 ± 3.1% and 60.1 ± 5.5%, respectively. The mean diameter of nanospheres encapsulated with ALM or both ALM and QS was 300 ± 123 nm and 294 ± 106 nm, respectively. The mean encapsulation efficiencies of ALM and QS in nanospheres were 71.7 ± 14.8 and 55.8 ± 23.1%, respectively.

The release profile of ALM and QS from nanospheres was also evaluated (Figure 2). The initial release of ALM was seen in the first 2 h of the study, in which 34 ± 1.3% of the ALM was released. This initial burst release was followed by a plateau with a mild slope. Finally, after 1 week the cumulative percentage of released ALM reached to 44.8 ± 0.8%. The same pattern of release was seen for QS, the initial burst release in the first 2 h was 25 ± 1%, which reached to 29.5 ± 0.2% after 1 week.

Lesion development after challenge
Lesion development was monitored by weekly measurement of footpad thickness.

Figure 1. Scanning electron micrograph of PLGA nanospheres encapsulated with ALM + QS
(Fig. 3). Encapsulation of ALM in PLGA nanospheres induced a significantly (P<0.05) smaller lesion size, compared with all the other groups, after week 4. In contrast, QS adjuvant induced a larger lesion size compared to the control groups. The largest lesion size was seen in mice immunized with (ALM+QS)PLGA. The lesion size was similar in mice immunized with (ALM)PLGA+QS or ALM or PBS. The results also showed that there was no significant difference (P>0.05) between mice immunized with ALM+QS, and (ALM)PLGA+QS and ALM.

**In vitro cytokine production by splenocytes**

The supernatant of cultured splenocytes were used to titrate the level of IFN-\(\gamma\) and IL-4 production. The levels of IFN-\(\gamma\) in the supernatant of SLA stimulated splenocytes in group of mice immunized with (ALM)PLGA+QS were significantly (P<0.0001, except for ALM+QS group) higher than the other groups, (ALM+QS)PLGA group showed an intermediate level of IFN-\(\gamma\) and the significantly (P<0.01) lowest levels of IFN-\(\gamma\) was seen in group of mice that received ALM.
The levels of IL-4 in the supernatant of SLA stimulated splenocytes in the mice that received either PBS or ALM were significantly (P<0.05) lower than all the other groups. There was no significant difference in the levels of IL-4 production between mice immunized with ALM+QS, (ALM)PLGA, ALM+QS, ALM or PBS. At week 3 after the last booster, the spleens were removed and the splenocytes were stimulated in vitro with SLA (10 mg/ml), concanavalin A (2.5 µg/ml), or with no stimulation (medium alone). Production of IFN-γ (A) and IL-4 (B) were assessed using sandwich ELISA on supernatants collected at 72 h of culture. Cells from 3 mice per group were pooled. The bars represent the mean and SEM of triplicate wells.

The levels of IL-4 in the supernatant of SLA stimulated splenocytes in the mice that received either PBS or ALM were significantly (P<0.05) lower than all the other groups. There was no significant difference in the levels of IL-4 production between mice immunized with ALM+QS, (ALM)PLGA, (ALM)PLGA+QS or (ALM+QS)PLGA (Figure 4).

**Antibody response**

In order to determine the type of immune response generated in immunized mice, the anti-SLA specific IgG, IgG1 and IgG2a antibodies were titrated before (Figs. 5A) and at week 14 after *L. major* challenge (Figs. 5B). As shown in Figure 5A, the significantly highest titer (P<0.05) of IgG2a and IgG1 (before challenge) were seen in sera of mice immunized with (ALM)PLGA+QS or ALM+QS. The significantly lowest IgG1 (P<0.0001) and IgG2a (P<0.05, except for (ALM)PLGA group titers were seen in mice immunized with ALM alone. The ratios of IgG2a/IgG1 in sera of mice immunized with ALM+QS or (ALM)PLGA+QS were significantly (P < 0.01) higher than all other groups (Figs. 5C). Mice immunized with (ALM)PLGA showed the significantly (P<0.05) lowest IgG2a/IgG1 ratio.

*D. Leishmania major* challenge induced elevation of IgG, IgG1 and IgG2a antibodies in all groups of mice compared with the antibody titers before challenge (Figs. 5B). All groups showed similar level of IgG1 antibodies. While before challenge a significantly (P<0.05) lowest IgG2a titers was seen in ALM group, after challenge this group showed the significantly (P<0.0001) highest IgG2a titers and IgG2a/IgG1 ratio compared to the other groups (Figs. 5C).

**DISCUSSION**

Vaccines composed of killed parasites (first-generation) induce low efficacies (54%), as tested in humans and dogs since 1940. Live genetically modified parasites, or bacteria or viruses containing *Leishmania* genes, recombinant or native fractions (second-generation vaccines) have been studied since the 1990s. The use of adjuvants increased vaccine efficacies of the purified antigens to 82%, in Phase III dog trials. Recombinant second-generation vaccines and third-generation DNA vaccines had an intermediate potential in parasite load reduction of 68% and 59% in laboratory animal models, respectively, but their success in field trials had not yet been reported (Palatnik-de-Sousa, 2008).

Therefore for formulation of an efficient vaccine, a proper immuno-adjuvant is needed. Among the immuno-adjuvants tested, IL-12 has been able to
induce protection against co-administered Leishmania antigens (Modabber, 1995). The efficacy of single and multiple doses of ALM mixed with BCG was checked in humans (Khamesipour et al., 2006; Noazin et al., 2008) and was able to induce partial protection in C57Bl/6 and Balb/c mice (Keshavarz Valian et al., 2008). CpG ODN as an immunomodulator adjuvant has also shown potential and could induce high immune responses and protection in mice (Jaafari et al., 2007).

Particulate drug delivery systems could act both as vaccine delivery systems and adjuvant and have been able to potentiate the immune responses against encapsulated antigens (Diwan et al., 2001).

To enhance the immunogenicity of ALM, PLGA nanospheres encapsulated with ALM alone or ALM plus QS were used to immunize susceptible Balb/c mice. The protection rate and the extent and type of immune response generated were evaluated in immunized mice.

Particulate antigens facilitate interaction with antigen presenting cells and induce a stronger immune response compared to soluble antigens (Gupta & Siber, 1995; Rebelatto et al., 2001). Therefore, in this study PLGA nanospheres were used to convert soluble antigens to particulate ones. Furthermore, the PLGA microspheres and nanospheres induce a Th1 type of response (Newman et al., 1998; Diwan et al., 2002).

At the present study nanospheres with the mean diameter of 294 nm were prepared. It has been shown that particles smaller than 10 microns in diameter could be taken up by macrophages and APCs (Ahsan et al., 2002). Therefore the prepared nanoparticles could be readily phagocytosed by macrophages.

In vitro release studies showed that most of the encapsulated antigens and adjuvants remain entrapped in the nanospheres and are not easily released from the particles. Therefore, most of the injected antigens and adjuvants are in particulate form and as such the stronger immune stimulation induced is due to of the encapsulated antigens.

Resistance or susceptibility to L. major infection depends upon generation of Th1 or Th2 responses, respectively. The key cytokine and antibody subtype indicative of Th1 responses are IFN-γ and IgG2a, whereas IL-4 and IgG1 are key indicators for Th2 response (Diwan et al., 2002; Sacks & Noben-Trauth, 2002; Shimizu et al., 2003).

Figure 5. Levels of anti-SLA-specific IgG, IgG2a, and IgG1 in sera of BALB/c mice immunized SC, 3 times in 3 weeks intervals, with (ALM+QS)PLGA, (ALM)PLGA+QS, (ALM)+PLGA, ALM+QS, ALM or PBS. Blood samples were collected at week 3 after the last booster (A) and at week 14 after challenge (B). The SLA-specific IgG, IgG2a and IgG1 levels were assessed using ELISA method. Panel C indicates the ratio of IgG2a/IgG1 based on absorbance. The assays were performed in triplicate at 200-fold dilution for each serum sample. Values are the mean ± SEM.

![Figure 5](image-url)
In group of mice immunized with (ALM)PLGA, progress of lesions induced by L. major infection, from week 4 after challenge, was significantly (P<0.05) slower compared to mice receiving ALM alone. It is an indication of protection induced by nanospheres. Encapsulated ALM resulted in a lower IgG2a/IgG1 ratio compared with ALM alone which indicates a Th2 response. However, the IFN-γ concentration (an indicator of Th1 response) in group of mice that received (ALM)PLGA was significantly (P<0.001) higher compared to the ALM group.

Previous studies on mice using PLGA nanospheres encapsulated with tetanus toxoid (TT) nanospheres efficiently induced a stronger immune response which were even stronger than conventional vaccine, alum absorbed TT. The IFN-γ titers induced with (TT)PLGA was 8 times more than the TT solution and about 2 times more than the alum-TT group. However, similar to the present study, the IgG2b/IgG1 ratio in nanospheric group was lower than the TT solution (Diwan et al., 2002).

QS as a natural adjuvant was also co-administered with ALM, both mixed or co-encapsulated. The results of different tests were indicative of an intermediate potential of QS. Before challenge, the ALM+QS group induced a higher IgG1, IgG2a titer, but the IgG2a/IgG1 ratio compared to the group received ALM alone was not significantly different (P>0.05). ALM+QS induced higher IFN-γ and IL-4 than ALM group (P<0.001). The protection induced with ALM+QS was more than for the ALM group (Fig. 3), but differences were not significant. The PLGA nanospheres were also mixed with QS solution. The (ALM)PLGA+QS could induce higher IgG2a/IgG1 ratio and IFN-γ and IL-4 levels, compared with (ALM)PLGA group. But in the presence of QS the greater lesion size was seen.

Co-encapsulation of QS with ALM in the nanospheres (ALM+QS)PLGA had a negative effect. In mice immunized with (ALM+QS)PLGA, the lesion size was more than (ALM)PLGA+QS group. The IgG2a/IgG1 ratio, IFN-γ and IL-4 levels was also decreased when QS was co-encapsulated with ALM. In another study, PLGA nanospheres co-encapsulated with tetanus toxoid and Quillaja saponins were used for nasal immunization of rabbits and induced higher serum IgG titers (P < 0.01) than nanospheres encapsulated with TT alone (TT)PLGA (Mohaghegh & Tafaghodi, 2010). For immunization against AIDS, when soluble QS21 was mixed with the encapsulated MN rgp120 in PLGA microspheres, the antibody titers were increased by a factor of 5 over the titers with encapsulated MN rgp120 alone. An additional fivefold increase in antibody titers was observed for guinea pigs immunized with co-encapsulated MN rgp120 and QS21 on the same microspheres (Cleland et al., 1994). In the above studies, co-encapsulation of Quillaja saponins with TT and MN rgp120 antigens in PLGA particles has increased immune responses, while at the present study co-encapsulation of QS with ALM in the nanospheres (ALM+QS)PLGA had a negative effect. Therefore, this negative effect could not be attributed to interaction of QS with PLGA polymer matrix. In another study, co-encapsulation of ALM and QS in alginate microspheres, similar to present study had a negative effect on immune responses (unpublished data). These could be possibly attributed to interaction of ALM and QS in particulate form, but more studies are needed to clarify the negative effect of co-encapsulation of ALM and QS.

These are several studies in which a mixed Th1 and Th2 immune responses have been reported for saponin adjuvants. Saponins have been found to enhance phagocytosis and stimulate secretion of cytokine such as IL-1, IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α. Such a broad range of cytokines is consistent with mixed Th1/Th2 responses (Song & Hu, 2009). Xie et al. (2009) used Platycodon grandiflorum saponin (PGS) as adjuvant for ovalbumin. After S.C. injection in mice, PGS showed a balanced Th1 and Th2 immunological response (Xie et al., 2009). For the immunotherapy of dogs, when Quil A was added to leishmune vaccine, average of the clinical scores decreased, but average of
CD4+ Leishmania-specific lymphocytes increased (Santos et al., 2007). P. notoginseng saponin elicited a Th1 and Th2 immune response in mice by regulating production and gene expression of Th1 and Th2 cytokines (Yang et al., 2007).

On the other hand, several authors have shown that Quillaja saponins (including QS-21) stimulated the production of CTLs and induced Th1 cytokines (IL-2 and IFN-γ) and antibodies of the IgG2a isotype to protein antigens (Kensil et al., 1995; Kensil 1996). The potential of Quil A in induction of Th1 immune responses against murine visceral leishmaniasis was demonstrated in their combination with the fucose-mannose ligand of Leishmania donovani (FML). It was shown that the QS-21/FML and Quil A/FML groups achieved the highest IgG2a response, while Quil A/FML developed the strongest delayed type of hypersensitivity (DTH) and QS-21/FML animals showed the highest serum IFN-γ concentrations among five kinds of adjuvants (Santos et al., 2002). Quil A or QS-21 could also elicit antigen-specific Th1 responses against Plasmodium falciparum, foot-and mouth disease and Measles virus in mice (Santos et al., 2002).

The type of immune response that is reported after immunization of saponin-adjuvanted vaccines (Th1 or mixed Th1/Th2) depends not only on the adjuvant itself, but also on the factors like the antigen, administration route and immunization schedule (Song & Hu, 2009).

The other explanation for different immune responses is related to lipophilic acyl side chain of saponins which is shown to be responsible for the remarkable stimuli for CTL production against exogenous proteins and instability under physiological conditions (Marciani et al., 2000). The spontaneous deacylation of saponins in aqueous solution (Marciani et al., 2001), could lead to production of the deacylated saponins. These deacetylated saponins are significantly less toxic and elicit Th2 responses while fail to stimulate either a lymphoproliferative response or the formation of CTL (Marciani et al., 2000).

In summary the results of this study showed that PLGA nanospheres as delivery system and adjuvant induces the Th1 immune response against ALM antigen but QS adjuvant induced a mixed Th1/Th2 immune response.

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