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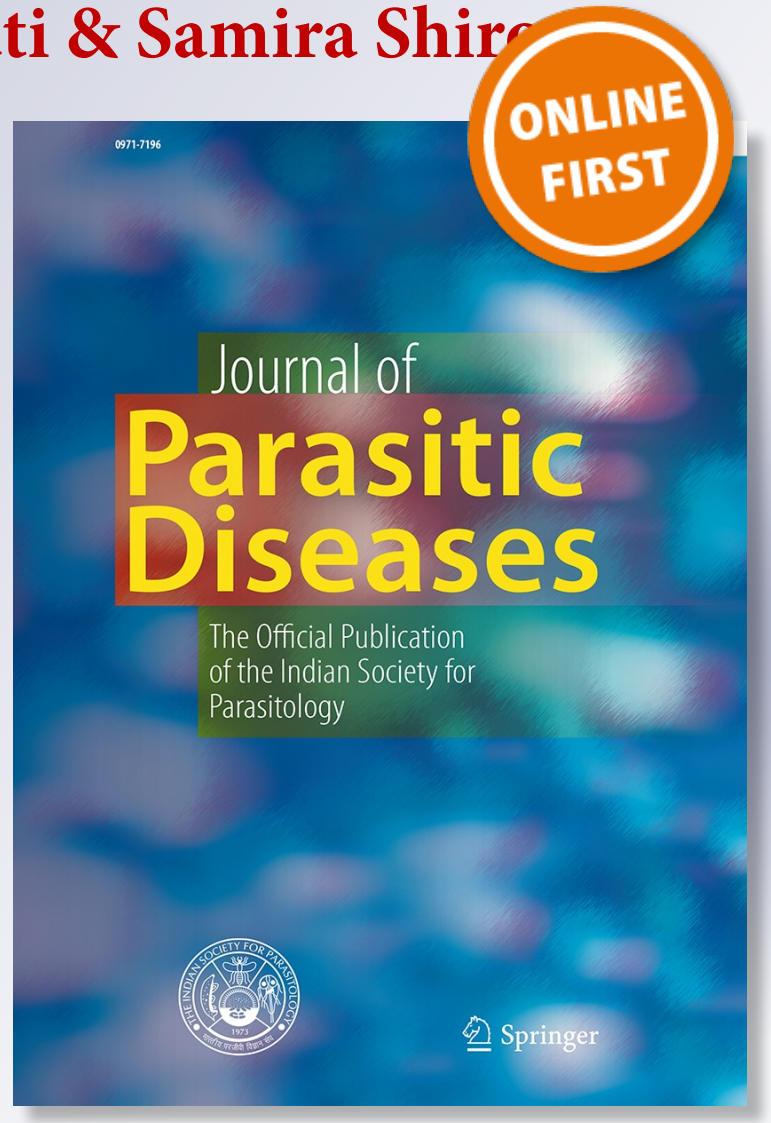

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REVIEW ARTICLE

Evaluation of the alum–naloxone adjuvant activity against experimental murine leishmaniasis due to *L. major*

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Abstract Leishmaniasis is caused by intracellular parasites of *Leishmania* species, which are transmitted by the bite of the sandfly. Recovery and protection against the infection depends on the induction of a strong Th1 type of immune response. Vaccination of mice with the opioid antagonist naloxone can promote the activation of the Th1 responses. We studied the efficacy of the mixture of naloxone and alum, as an adjuvant, to enhance immune responses and induce protection against *Leishmania major* infection in BALB/c as a susceptible mouse model. BALB/c mice were immunized with Ag–naloxone–alum, Ag–alum, Ag–naloxone or PBS subcutaneously three times at 2-week intervals. The humoral and cellular specific immune responses were assessed 2 weeks after the last immunization and compared with the control mice. Our results indicated that the administration of alum–naloxone as an adjuvant increased the capability of *L. major* promastigote antigens to enhance lymphocyte proliferation, the levels of IFN-γ, and the IFN-γ/IL-5 ratio. The results of DTH showed that there were no significant differences in footpad swelling between the groups of immunized mice as compared with the non-vaccinated control group; however, no significant differences were observed in the survival rate among groups. It can be concluded that although immunization with the alum–naloxone mixture in combination with the autoclaved *L. major* promastigote antigens could enhance cellular immunity and shift the immune response to a Th1 pattern, it could not protect the mice against *Leishmania major* infection.

Keywords *Leishmania major* · Alum · Naloxone · Adjuvant

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Introduction

Leishmaniasis is caused by intracellular parasites of Leishmania species, which are transmitted by the bite of the sandfly. This disease has a wide range of clinical symptoms from chronic cutaneous lesions to visceral leishmaniasis, which is fatal if left untreated (Singh and Sundar 2012). Currently, 350 million people in 88 countries are at risk of infection and 14 million people are infected (Nagill and Kaur 2011). Recovery and protection against the infection depends on the induction of a strong

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Th1 type of immune response (Sukumaran and Madhubala 2004). The activation of Th1 responses is associated with the production of cytokines such as IFN-γ and IL-12 which stimulate the microbicidal activity of macrophages (Ameen 2010). Exposure to opioids increases the susceptibility to bacterial, fungal, and opportunistic infections (Friedman et al. 2003; Roy et al. 2011). It is now clear that the endogenous opioids modulate both innate and acquired immune responses (McCarthy et al. 2001). Naloxone (NLX), as an opioid antagonist, blocks μ-opioid receptors and reduces the positive reinforcing effects of opioids (Panerai et al. 1995). NLX has been approved by Food and Drug Administration (FDA) for the treatment of opioid addiction (www.accessdata.fda.gov). There are experiments showing that naloxone can enhance lymphocyte proliferation and shift the immune response from Th2 to Th1 in murine model (Sacerdote et al. 1998, 2000). Furthermore various studies have shown that naloxone is capable of inducing protective immune responses when used as an adjuvant in combination with different pathogens, such as *Salmonella typhimurium*, *Listeria monocytogenes*, and *Herpes simplex virus type 1* (HSV-1), indicating the ability of naloxone as an adjuvant (Jamali et al. 2007; Jazani et al. 2010; Jazani et al. 2011). On the basis of these findings, we evaluated the immunogenicity potential of heat-killed *Leishmania major* promastigotes in combination with the mixture of alum and naloxone as an adjuvant against *Leishmania major* infection in BALB/c as a susceptible mouse model.

Materials and methods

Six to eight week-old female BALB/c mice were purchased from Razi Vaccine and Serum Research Institute, Iran. Experiments were approved by the Ethics Committee of Urmia University of Medical Sciences.

Parasites and sample preparation

The *L. major* strain MRHO/IR/75/ER was provided from the School of Public Health, University of Tehran. The virulence of the parasite was maintained by passage in the BALB/c mice. Promastigotes of *L. major* were harvested in the stationary growth phase by centrifugation (2000*g*, 20 min, 4 °C), and washed 3 times with cold PBS (pH 7.2). The parasites were homogenized in PBS and then the cell lysate was autoclaved. The protein concentration was determined by the Bradford method. Antigen preparations were kept in small aliquots at −20 °C until use. Naloxone (Sigma, Germany) at a concentration of 6 mg/kg was dissolved in PBS. Each dose contained 40 μg/ml of parasite proteins.

Immunization of BALB/c mice

All the mice were injected with a total volume of 150 ml. Different groups of mice (10 per group) were vaccinated subcutaneously three times at 2-week intervals with either one of the following formulations: Vac Group (50 μl Ag + 100 μl PBS/mouse), Group Al–Vac (50 μl Ag + 50 μl Al + 50 μl PBS/mouse), NLX–Vac Group (50 μl Ag + 50 μl NLX + 50 μl PBS/mouse), and AL–NLX–Vac Group (50 μl Ag + 50 μl Al + 50 μl NLX/mouse). The control group received 150 μl of PBS.

Lymphocyte proliferation test

The mice (five mice from each group) were sacrificed at 2 weeks after the last immunization. Their spleens were aseptically removed and homogenized in incomplete RPMI 1640 (without FBS). The erythrocytes were disrupted using ammonium chloride (0.9 %); then, the spleen cells were washed three times with incomplete RPMI 1640. The cells were cultured in duplicate at 1×10^5 cells per well in complete RPMI (Gibco-BRL) containing 10 % FBS (Gibco-BRL), 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 IU/ml penicillin. One hundred microliters of diluted cell suspension were dispensed into 96-well flat-bottom culture plates. Splenocytes were incubated at 37 °C in 5 % CO_2 for 48 h with or without addition of *L. major* antigen. Lymphocyte proliferation was measured by an MTT assay [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl-blue, Sigma, Germany].

Cytokines analysis

Supernatants were collected after 62 h of incubation from cell cultures and the levels of cytokines IFN-γ and IL-5 were measured by a commercial ELISA kit (ELIZA PRO kit for Mouse IFN-γ, IL-5, MabTech).

Delayed type hypersensitivity (DTH) assessment

Three week after the last vaccination, the mice were inoculated in the left footpad with 1×10^7 metacyclic promastigotes of *L. major* in a volume of 50 μl. Their right footpads were inoculated with the same volume of PBS as the control group. Metacyclic forms were previously isolated from stationary cultures, washed in PBS, and freeze-thawed (three times) and resuspended in PBS for inoculation. Footpad swelling was measured with a metric caliper (thickness of the left footpad in comparison to thickness of the right footpad).

Challenge with live promastigotes of *L. major*

Mice (five animals per group) were challenged subcutaneously in the tail base with 1×10^6 stationary phase

promastigote (MRHO/IR/75/ER) in a volume of 50 µl, 4 weeks after the last immunization. Lesion development and survival rates of the vaccinated and control mice were monitored for 20 days post challenge.

Statistical analysis

All experiments were analyzed by variance analysis (ANOVA) followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Result

Lymphocyte proliferation

Two weeks after the last immunization, cell proliferation in response to *L. major* antigen with various adjuvants was evaluated. The mean level of MTT in the mice immunized with Al–NLX–Vac and NLX–Vac induced significantly more lymphocyte proliferation when compared to the control groups ($P < 0.01$ and $P < 0.05$, respectively). Furthermore, lymphocyte proliferation was significantly more in the mice immunized with the Al–NLX–Vac compared to lymphocyte proliferation in mice that received Al-Vac and Vac ($P < 0.01$ and $P < 0.05$, respectively). There was no significant difference in lymphocyte proliferation between other groups (Fig. 1).

In vitro cytokine production by splenocytes

To determine cell-mediated immunity, the supernatant of the cultured splenocytes was analyzed for the level of IFN-γ, IL-5 cytokines using the ELISA method. As shown in Fig. 2, all groups of the immunized mice induced significantly higher levels of IFN-γ production when compared to the control groups ($P < 0.001$). The levels of IFN-γ in the supernatant splenocytes in groups of immunized mice with Al–NlX–Vac were significantly higher than Vac groups ($P < 0.\ 01$). Furthermore, a significant difference was observed in IFN-γ response between NlX–Vac and Vac groups ($P < 0.05$). The IFN-γ/IL-5 ratio in the supernatant of the cells of the mice immunized with Al–NlX–Vac was higher than other groups but there was no significant difference in any of the groups.

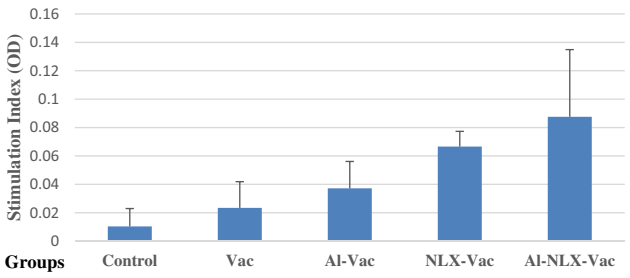

Fig. 1 Lymphocyte proliferation response to the Al–NLX vaccine in immunized mice. Two weeks after the last immunization, the spleen cells were stimulated with parasite antigen. After 48 h of in vitro stimulation, the lymphocyte proliferation was evaluated using an MTT assay. Stimulation indices (SI) were determined and expressed as differences between the absorbance of treated and untreated wells. The values are mean ± SE ($n = 5$ mice per group)

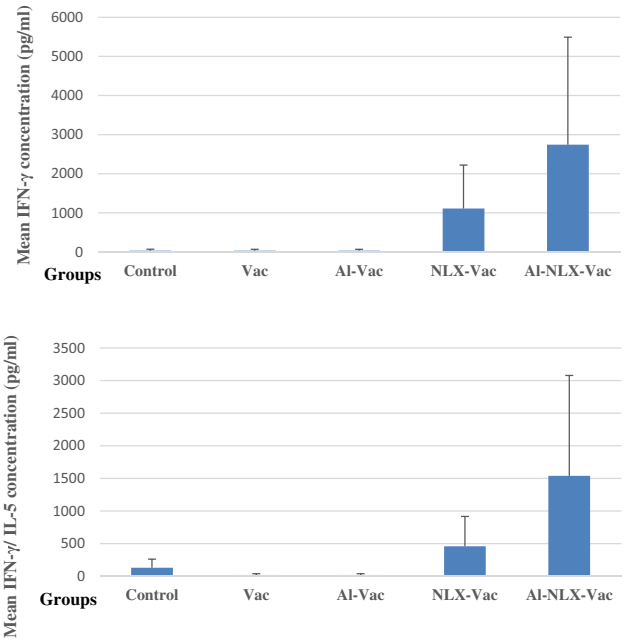

Fig. 2 Effect of administering alum–NLX mixture on IFN-γ production and IFN-γ/IL-5 ratio. Two weeks after last immunization the levels of IFN-γ (**a**) and IFN-γ/IL-5 ratio (**b**) were measured by ELISA in the supernatants of cells cultures from five mice per group. Cells were stimulated in vitro for 72 h with 5 µg/ml of parasite antigen. The values are mean ± SE ($n = 5$ mice per group)

Delayed type hypersensitivity responses (DTH)

Induction of the DTH reaction based on the cell-mediated response to parasite antigens was monitored in all groups. The results of DTH showed that there were no significant differences in footpad swelling between the groups of immunized mice as compared with the non-vaccinated control group (Fig. 3).

Challenge results

The results of lesion development and survival rate showed that there was no significant difference among mice groups 10 weeks after challenge with *L. Major* (data not shown).

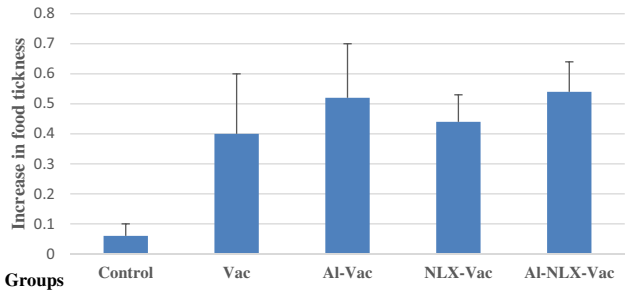

Fig. 3 DTH response to parasite antigens in BALB/c mice immunized with different adjuvants. Three weeks after last immunization 1×10^7 freeze-thawed *L. major* promastigotes and PBS in a final volume of 50 µl was injected in left and right footpad of mice respectively. The difference in footpad swelling was measured at 72 h

Discussion

Cutaneous leishmaniasis due to *L. major* is a self-limiting disease in humans that leads to life-long immunity against reinfection. However, in spite of the efforts to formulate a leishmaniasis vaccine during the last decade, an effective vaccine is not yet available. This failure is likely due to lack of an appropriate adjuvant (Badiee et al. 2007).

Molla Hassan et al. (2009) suggested that naloxone was an effective immunoadjuvant in cancer immunotherapy and could improve anti-tumor immunity by reducing the CD4+ CD25+ Foxp3+ regulatory T cells. Previous investigations have shown that vaccination based on an opioid antagonist as an adjuvant such as naloxone or naltrexone (which are structurally similar pure opiate receptor antagonists but it has a longer biologic half-life) and propranolol (as Beta-Adrenergic Receptor Antagonist) could improve protection against various intracellular infections in BALB/c mice via modulation in the Th1/Th2 balance or affecting macrophages through binding to Toll-like receptors (Jazani et al. 2010, 2011; Molla Hassan et al. 2009; Mohammadzadeh Hajipirloo et al. 2014; Shahabi et al. 2014). Furthermore, it has been shown that in mice and humans, naloxone increases T lymphocytes proliferation, increases the NK cells activity, and accelerates local inflammation via a direct effect on monocytes, macrophages and dendritic cells.

In this study, the alum–naloxone mixture, as a Th1 immunostimulatory adjuvant, was used with autoclaved *L. major* to elicit a protective immune response challenge with the parasite in susceptible BALB/c mice.

Our results showed that immunization with Al–NLX–Vac stimulated the proliferation of spleen cells significantly. Furthermore, footpad swelling after infection with *L. major* was measured and the results demonstrated that Al–NLX–Vac elicited strong DTH responses.

The Al–NLX–Vac group induced higher IFN-γ, lower IL-5, and a higher IFN-γ/IL-5 ratio when compared with other groups, which is an indication that alum–naloxone mixture in combination with the autoclaved *L. major* promastigotes antigens skews the immune response towards the Th1 response. The shift to the Th1 response plays a major role in the induction of cellular immunity. These results are in agreement with other studies showing that NLX induces the Th1 type of immune response (Manfredi et al. 1993; Sacerdote et al. 1996).

Contrary to expectations, there was no significant difference in the death rate until 12 weeks after infecting the mice immunized with naloxone, antigen alone, alum–naloxone, AL–antigen, or buffer. One reason could be the administration of low dose naloxone for vaccination. It is speculated that naloxone has a dose-dependent biphasic effect on microorganisms. In our study, naloxone at a concentration of 6 mg/kg was used for vaccinating the mice against *L. major* infection. Karaji and Hamzavi (2012) reported that the administration of high dose (5 mg/kg) naloxone improved protection against *L. major* infection and treatment of mice with a dose of 5 mg/kg did not have a significant effect on the infection. In contrast, low-dose naltrexone, an antagonist at the mu-opioid receptor, has been showed to have immunomodulatory and antitumor effects (Ebrahimpour et al. 2013). Accordingly, it seems that more research is needed for determining the effective dose of naloxone for vaccination.

Conclusion

The data reported here provided evidence that administration of the alum–naloxone mixture as an adjuvant in combination with the autoclaved *L. major* promastigotes antigens could improve the protection against *L. major* infection in BALB/c mice to some degrees, presumably through modulating the Th1/Th2 balance. However, more research is required to elucidate the details of the alum–naloxone mixture effect on immune cells.

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Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest.

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