Alexandria Journal of Medicine

ISSN: 2090-5068 (Print) 2090-5076 (Online) Journal homepage: https://www.tandfonline.com/loi/tajm20

Comparison of immunogenical potency of *Leishmania major* (MRHO/IR/75/ER) antigens prepared by 3 different methods in conjunction with Alum-Naltrexone adjuvant in BALB/c mice

Peyman Khezri, Shahram Shahabi, Esmaeil Abasi & Habib Mohammadzadeh Hajipirloo

To cite this article: Peyman Khezri, Shahram Shahabi, Esmaeil Abasi & Habib Mohammadzadeh Hajipirloo (2018) Comparison of immunogenical potency of *Leishmania major* (MRHO/IR/75/ER) antigens prepared by 3 different methods in conjunction with Alum-Naltrexone adjuvant in BALB/c mice, Alexandria Journal of Medicine, 54:4, 503-510, DOI: 10.1016/j.ajme.2018.10.004

To link to this article: https://doi.org/10.1016/j.ajme.2018.10.004

Published online: 17 May 2019.

Alexandria Journal of Medicine 54 (2018) 503–510

HOSTED BY

Contents lists available at ScienceDirect

Alexandria Journal of Medicine

journal homepage: http://www.elsevier.com/locate/ajme

Original Article

Comparison of immunogenical potency of *Leishmania major* (MRHO/IR/75/ER) antigens prepared by 3 different methods in conjunction with Alum-Naltrexone adjuvant in BALB/c mice

Peyman Khezri [a], Shahram Shahabi [b], Esmaeil Abasi [a], Habib Mohammadzadeh Hajipirloo [a,*]

[a] Cellular and Molecular Research Center, Department of Parasitology and Mycology, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[b] Cellular and Molecular Research Center, Department of Immunology, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran

ARTICLE INFO

Article history:
Received 16 February 2018
Revised 5 August 2018
Accepted 11 October 2018
Available online 7 December 2018

Keywords:
Leishmania major
Immunization
Alum
Naltrexone
Opioid-receptor

ABSTRACT

Objectives: To evaluate the immunization ability of three *Leishmania major* antigens including formaline killed promastigotes (FKP), autoclaved *Leishmania major* (ALM) and soluble *Leishmania* antigen (SLA), they were used to immunize BALB/c mice in association with a mixture of alum and naltrexone (Alum-Nalt) as adjuvant.

Methods: Each mouse from any of three groups received FKP, ALM or SLA antigens. Three additional groups were injected with same antigenes plus Alum-Nalt. One more group was injected with PBS to be the control group. Booster injections were given at 14th and 28th days. Two weeks after the last immunization, seven mice from each group were exposed to live promastigotes subcutaneously, and skin lesion formation among each mouse was monitored and recorded for 60 days. One week later, cellular and humoral immune responses of other immunized mice were evaluated by measuring the serum levels of anti-ALM-specific IgG1, IgG2a and total IgG antibodies, splenic anti-ALM-specific IFN-γ and IL-5 production and splenic lymphocyte proliferation after adding ALM.

Results: In the challenge test, all mice immunized with ALM antigen and Alum-Nalt were prevented from formation of skin ulcer. Also, the mice from the same group showed higher IFN-γ production and splenic lymphocyte proliferation and higher anti-ALM-specific IgG2a production.

1. Introduction

Zoonotic cutaneous leishmaniasis (ZCL) is caused by the intracellular parasite *Leishmania major* and is transmitted by the bite of sand flies. The disease is endemic in more than 88 countries with about 12 million infected people. The incidence of cutaneous leishmaniasis is approximately 1.5–2 million people per year and it is one of the WHO's controlling priorities. Clinical signs are variable depending on the host immune responses.[1–4] Any of measures to prevent leishmaniasis including vector and reservoirs control and treatment procedures, have their own limitation. It seems that immunization of individuals at risk is the most effective prevention method.[5] Immunization against leishmaniasis includes the use of killed, molecular or fractionated vaccines, and leishmanization.[6,7] The goal of vaccination is to create effective and long-lasting immune responses against the infection. Subunit vaccines have been used, but for enhancing the efficacy, it is necessary to be used in association with adjuvants.[8–10] Because *Leishmania* is an intracellular parasite, cellular immune responses are required for diseases control.[11] Therefore, the antigen and adjuvant that are selected for vaccination against leishmania, should be able to induce cell mediated immunity responses. Alum is the only adjuvant that is authorized by FDA for use in human vaccines.[12] Alum acts by creating a source of antigen at the injection site and activation of complement, eosinophils and macrophages. Unfortunately, alum salts are relatively weak adjuvants and stimulate humoral immune responses. Also, when alum is used intradermal or subcutaneously, allergic reactions and granuloma formation can be seen at the injection site.[13,14] Using opioid antagonists as adjuvant can shift the immune response to Th1 profile.[15,16] Our previous studies have revealed that naltrexone is an opioid receptor antagonist and is able to activate Th1 and suppress Th2 cytokines via elimination of endogenous opioid peptides effects and therefore the use of naltrexone as an adjuvant in a vaccine model, can shift the immune response to Th1 profile and result in enhanced immunogenicity of the vaccine.[17,18] In this study a mixture of alum and naltrexone

Peer review under responsibility of Alexandria University Faculty of Medicine.
* Corresponding author.
E-mail address: h.mohammadzadeh@umsu.ac.ir (H.M. Hajipirloo).

https://doi.org/10.1016/j.ajme.2018.10.004
2090-5068/

was used as adjuvant in combination with three different *Leishmania major* antigens including formalin killed promastigote antigen (FKP), autoclaved *Leishmania major* (ALM) and soluble *leishmania* antigen (SLA) to evaluate and also compare the immunogenicity potential of each antigen in inducing cellular immune responses.

2. Materials and methods

2.1. Animals

Six to eight weeks old female inbred BALB/c mice were purchased from Razi Institute of Iran and divided randomly to seven groups, each group containing 20 mice. All experiments were done following the protocol that was approved by the Institutional Animal Care and Use at the Urmia University of Medical Sciences, Urmia, Iran.

2.2. Preparation of autoclaved Leishmania major (ALM) antigen

ALM preparation was performed according to a previously described method.[19] Briefly, The MRHO/75/IR/ER strain promastigotes of *Leishmania major* were cultured in RPMI 1640 including 15% fetal bovine serum (complete culture medium or CCM) and incubated at 24 °C in a shaking refrigerated incubator. Cultured promastigotes were harvested at stationary growth phase and were washed five times with sterile PBS (pH = 7.2) by centrifugation at 2000*g* for 15 min at 4 °C. The sediment was homogenized with glass-glass homogenizer on ice for 20 min and was autoclaved at 121 °C. The protein concentration was assayed by the Biuret chemical method and the sediment was stored at −20 °C until use for mice immunization.

2.3. Preparation of formalin killed promastigotes (FKP) antigen

Promastigotes in stationary phase were washed three times with cold PBS and 10 ml of 0.1% formalin was added to the sediment and the suspension was kept overnight at 4 °C and washed with PBS three times by centrifugation at 2000*g* for 15 min at 4 °C. Formalin killed promastigotes were counted and adjusted to 10^7 promastigotes/50 μl PBS. The prepared antigen was stored at −20 °C until use. To make sure that all promastigotes have been killed, sediment was added to CCM and placed in a shaking incubator for two weeks and was examined under the microscope.[20]

2.4. Preparation of soluble leishmania major antigen (SLA)

Promastigotes in stationary phase were washed three times with cold PBS and were counted and adjusted on 2×10^7/milliliter. Freeze/thawed was performed five times at −70 °C and room temperature and as described above suspension was homogenized on ice and centrifuged at 10,000*g* for 120 min at 4 °C. The supernatant was concentrated by dialyzing against polyethylene glycol (Sigma, D0655). The dialyzed fluid was sterilized by passing through a 0.22-μ filter. The protein concentration was assayed by the Biuret chemical method and the sediment was stored at −20 °C until use for mice immunization.

2.5. Immunization

Each member of mice groups received subcutaneously the specified injection doses as shown in Table 1 on the 1st, 14th, and 28th days.

2.6. Lymphocyte proliferation assay (MTT)

Three weeks after the last immunization, six to seven mice from each group were anesthetized by peritoneal injection of 50 μl of a mixture containing three volumes of PBS, two volumes of ketamine and one volume of xylazine. Blood samples were collected by cardiac puncture and serum samples were separated and stored at −20 °C until use for determining anti-ALM specific antibodies. Euthanasied by cervical dislocation, mice spleens were removed and crushed in RPMI 1640 without FBS (PCM) in the glass-glass homogenizer. The suspension was centrifuged at 2000 rpm for 5 min. The sediment was solved in 2 ml of PCM and 13 ml of 0.9% ammonium chloride was added for RBCs to be lysed. The tube was centrifuged for 5 min and the splenic cells were washed twice with PCM. Two milliliters of CCM was added to the sediment and viable cells were counted, using trypan blue dye, and were diluted to 10^5 cells in 100 μl of CCM. The cells were dispensed in 96-well culture micro plates (each mouse's splenocytes were plated in duplicate). The cells of antigen wells were stimulated with 20 μl of ALM antigen, and the volumes were adjusted to 100 μl per well and 100 μl of RPMI medium was added to control wells). Microplates were incubated at 37 °C with 5% CO_2 in moist condition for 48 h. Then, 20 μl of 5 mg/ml MTT solution [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide].[1–4,13,21] (sigma:M5655-1 g) was added to all test and control wells. Incubation was performed again in the same condition for 4 h. The microplates were centrifuged at 2000*g* for 10 min and the supernatant was removed from each well. For solubilizing the resulting crystals, 100 μl of DMSO was added to each well. Optical density (OD) was measured at 540 nm by ELISA reader. The differences between optic densities of antigen full wells and antigen free wells were calculated for each mouse of various groups, and the obtained values were recorded as the result of MTT test.[22]

2.7. Cytokine assays

Splenic cells were cultured in the same way that described in the previous phase except the longer incubation time (72 h). After centrifuging, the supernatant was aspirated from each well and stored at −70 °C until use for splenic IL-5 and IFN-γ assays using ELISA kit[23])Mouse IFN-γ ELISA development kit, MABTECH, Product code: 3321-1H-20 and Mouse IL-5 ELISA development kit, MABTECH, Product code: 3391-1H-6).

2.8. Antibodies assays

Using the serum sample that were secluded in the lymphocyte proliferation test, Anti-ALM specific total IgG, IgG1 and IgG2a antibodies were measured among different experimental mice groups. An aliquot of 100 μl of 10 μg/ml concentration of ALM in 0.05 M carbonate-bicarbonate buffer was add to each of 96 well microplates and incubated at 4–8 °C for overnight. Wells were washed and were blocked by adding 1% BSA in PBS. After 1 h at room temperature and washing, diluted serum samples (1/1000 in PBS) were added in duplicate to microplate wells. Incubation was performed at room temperature for 2 h. After washing, a diluted goat anti-mouse IgG1 (AbD Seroteck), IgG2a (AbD Seroteck) or total IgG (RayBiotech) peroxidase conjugates were added and microplates were incubated again for 2 h. Washing was performed and 100 μl of freshly prepared 3,3′,5,5′-Tetramethylbenzidine (TMB)(sigma: T2885-1G) was added to each well. After incubation, reaction was stopped by adding 1 N H_2SO_4 and optic densities for each subclass were read by ELISA reader at 450 nm and the differences between the obtained values for various groups were compared to evaluate the increase in antibody levels.

Table1
Different groups specified subcutaneous injection doses.

Injections						
Group name	SLA (μl)	FKP (μl)	ALM (μl)	Nalt. (μl)	ALUM (μl)	PBS (μl)
SLA-Alum-Nalt.	50	–	–	50	50	–
FKP-Alum-Nalt.	–	50	–	50	50	–
ALM-Alum-Nalt.	–	–	50	50	50	–
ALM	–	–	50	–	–	100
SLA	50	–	–	–	–	100
FKP	–	50	–	–	–	100
Control	–	–	–	–	–	150

2.9. Live parasite challenge

Two weeks after the last immunization, seven mice from each group were exposed to 10^6 live promastigotes, subcutaneously on the base of mouse tail, and the mice were monitored for appearance of ulcer at the injection site for two months.

2.10. Statistical analysis

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

3. Theory/calculation

The immune response to various antigens can be different if the antigens are mixed with the adjuvants. Protozoan parasite, Leishmania, has different antigens and there are various methods to provide these antigens for immunization and the immune response can be different depending on antigens. So, identification of more immunogenic antigens for each parasite and the needed type of immune response (cellular and or humoral) to inhibit or reduce the parasite pathogenicity, can facilitate the prevention of the disease via vaccination. Therefore, in the present study, with the aim of stimulating cellular activity, we used three *Leishmania major* antigens including FKP, ALM and SLA to evaluate their immunization ability in association with Alum-Naltrexone (Alum-Nalt) mixture as an adjuvant with a potential to stimulate the cellular immune response.

4. Results

4.1. Lymphocyte proliferation assay (MTT)

Optical density values obtained from the mice splenocytes stimulation by ALM antigen, are shown in Fig. 1. The lymphocyte

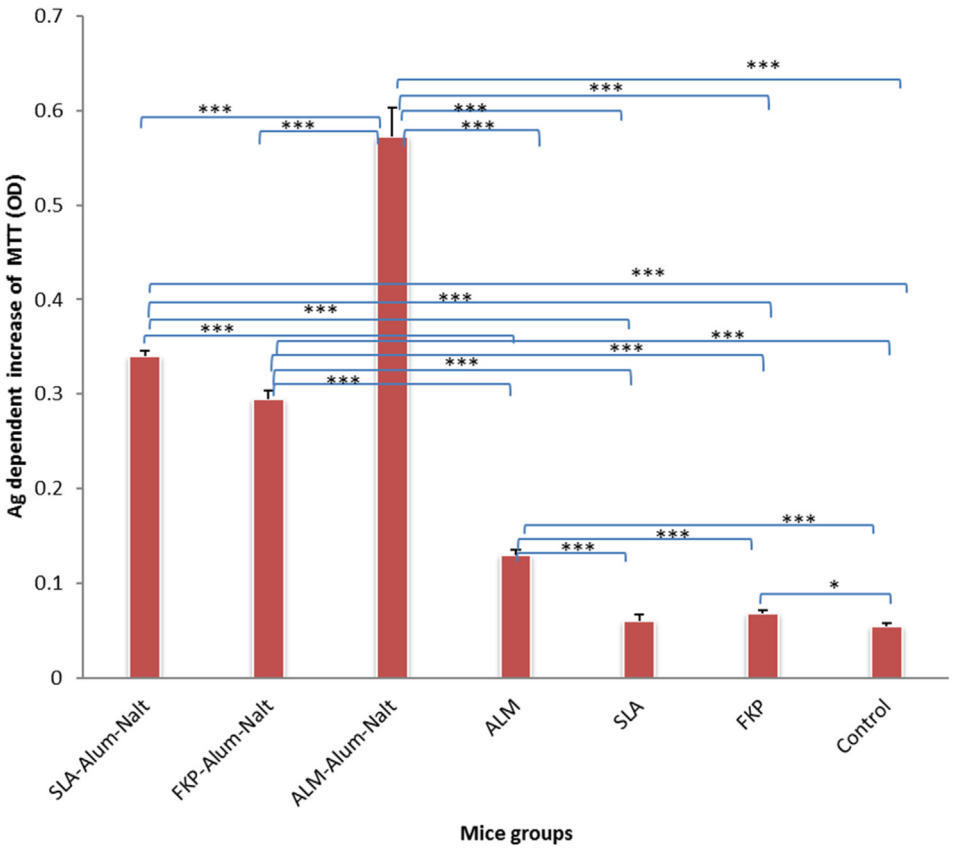

Fig. 1. Effect of immunization of mice with ALM, FKP or SLA antigens with or without ALUM-NALT mixture as adjuvant on splenic lymphocytes proliferation on exposure to ALM antigen. (*: p value < 0.05/**: p value < 0.01/***: p value < 0.001).

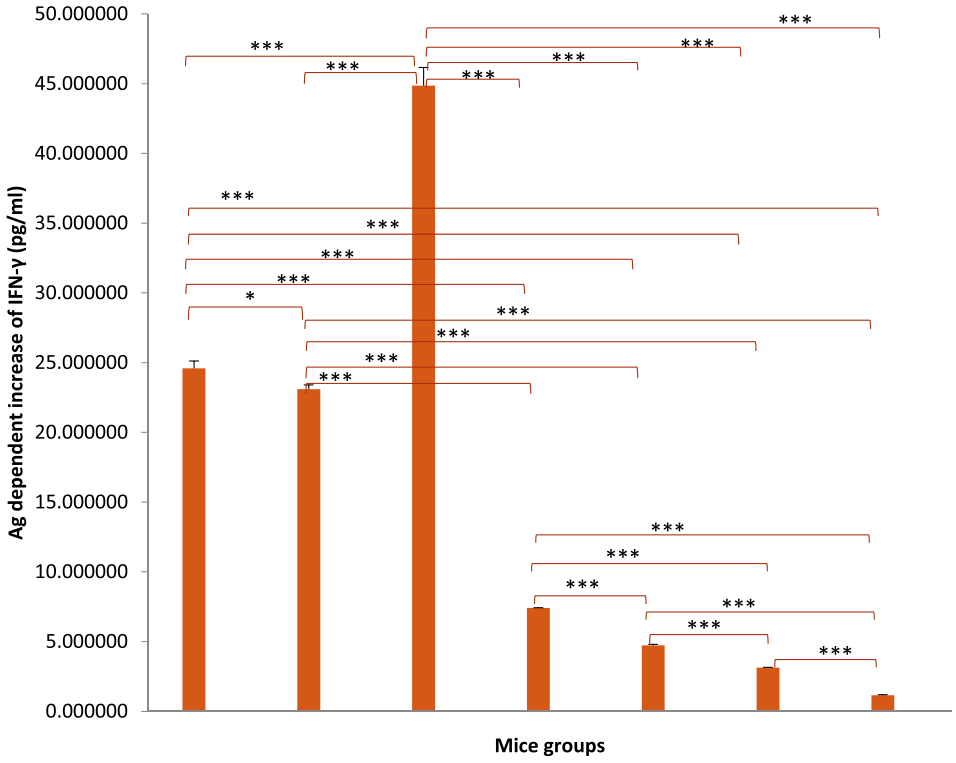

Fig. 2. Effect of immunization of mice with ALM, FKP or SLA antigens with or without ALUM-NALT mixture as adjuvant on the splenic IFN-γ production on exposure to ALM antigen. (*: p value < 0.05/**: p value < 0.01/***: p value < 0.001).

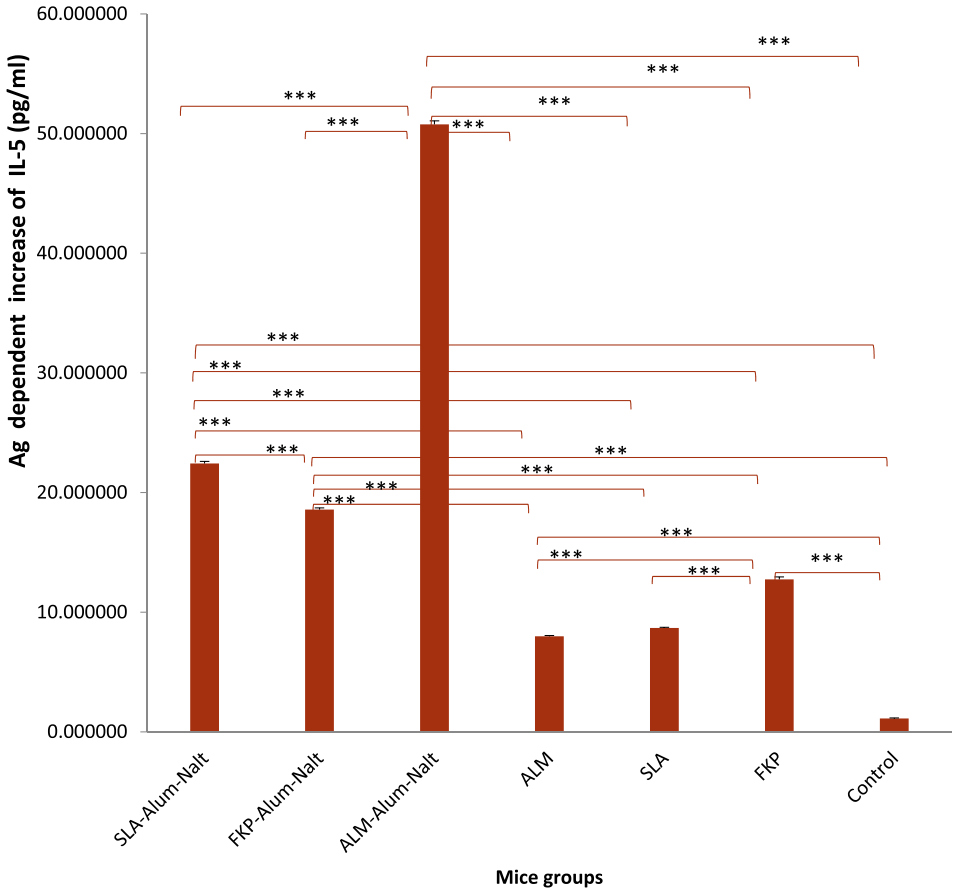

Fig. 3. Effect of immunization of mice with ALM, FKP or SLA antigens with or without ALUM-NALT mixture as adjuvant on the splenic IL-5 production on exposure to ALM antigen. (*: p value < 0.05/**: p value < 0.01/***: p value < 0.001).

proliferation response, in the groups receiving the antigen plus adjuvant were significantly higher in comparison with adjuvant free and control groups. Maximum increasing was observed in the ALM-Alum-Nalt group ($p < 0.001$).

4.2. IFN-γ assay

The value of released IFN-γ in adjuvant receiving groups was higher than other groups and the use of adjuvant led to an increased splenic lymphocytes response in dealing with ALM antigen (Fig. 2). Splenic lymphocytes of ALM-Alum-Nalt group produced much more IFN-γ in comparison with other groups when exposed to ALM antigen ($p < 0.001$).

4.3. IL-5 assay

Fig. 3 shows the results of splenic IL-5 production among different mice groups. The value of splenic IL-5, was higher in adjuvant receiving groups in comparison with those groups that had not received adjuvant. This increasing, in the group receiving ALM-Alum-Nalt, was more than all other test and control groups ($p < 0.001$).

4.4. IgG subclasses assay

4.4.1. Total IgG

Adjuvant receiving mice groups produced more anti-ALM specific total IgG antibody than adjuvant free and control groups and the highest level was seen in the ALM-Alum-Nalt. Group (Fig. 4) ($p < 0.001$).

4.4.2. IgG1 assay

As is shown in Fig. 5, the value of anti-ALM-specific IgG1, was higher in adjuvant receiving groups in comparison with no adjuvant groups, and this increasing, in the group receiving ALM-Alum-Nalt., is more than all other test groups and control group ($p < 0.001$).

4.4.3. IgG2a assay

The results of anti-ALM-specific IgG2a assay is shown in Fig. 6. The value of anti-ALM specific IgG2a, was higher in adjuvant receiving groups in comparison with adjuvant free groups and this increasing, in the ALM-Alum-Nalt. group, was more than other test and control group. So, ALM-Alum-Nalt group, has more statistically significant difference with other test groups and control group ($p < 0.001$).

4.5. Challenge test

No ulcer production was observed in the seven mice that were exposed to live promastigotes in ALM-Alum-Nalt group. The percentage of skin lesions production among the other mice groups, are as follows: SLA-Alum-Nalt: 28.57% (2 out of 7 mice), FKP-Alum-Nalt: 28.57% (2 out of 7 mice), ALM without adjuvant: 42.85% (3 out of 7 mice), SLA without adjuvant: 57.14% (4 out of 7 mice), FKP without adjuvant: 66.66% (4 out of 6 mice. One of

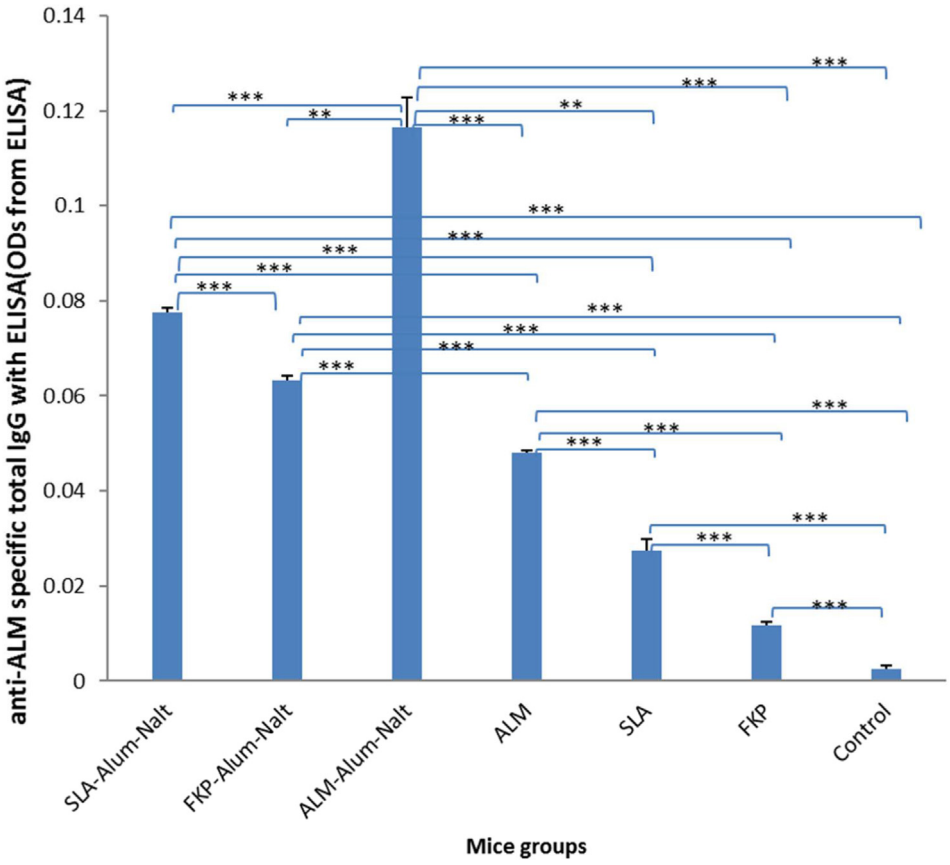

Fig. 4. Comparison of optical densities from ELISA test for serum anti-ALM-specific total IgG among different groups of mice. (*: p value < 0.05/**: p value < 0.01/***: p value < 0.001).

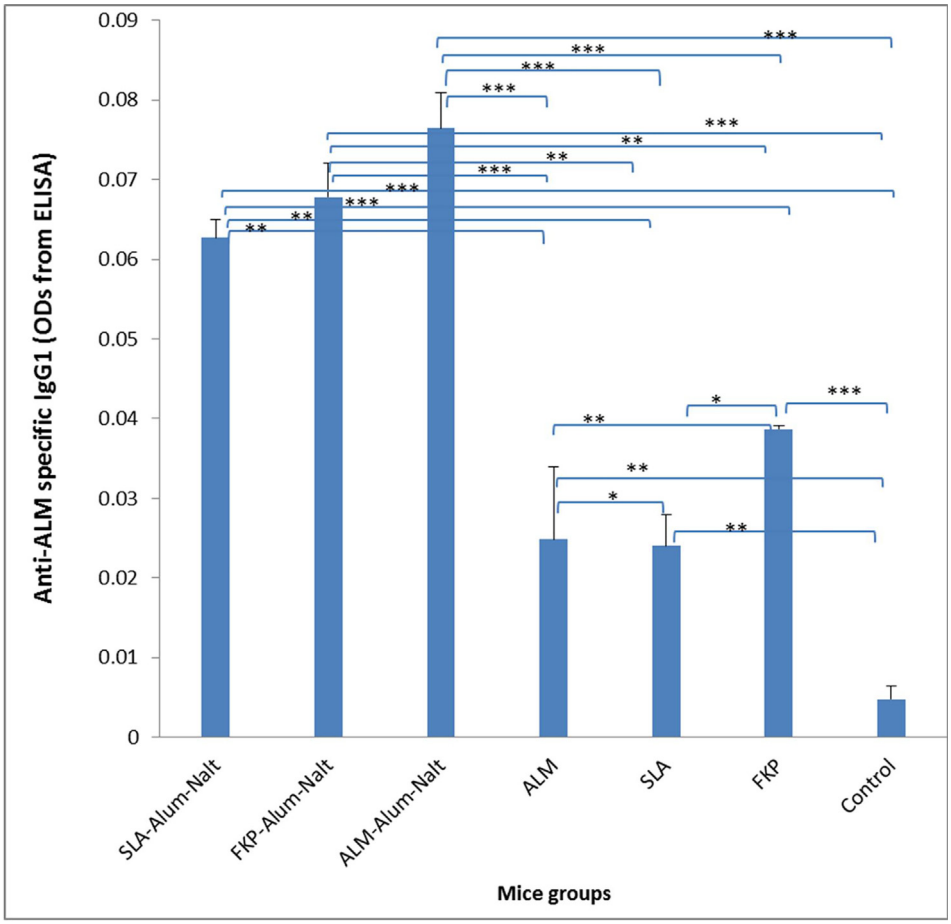

Fig. 5. Comparison of optical densities from ELISA test for serum anti-ALM-specific IgG1 among different groups of mice. (*: p value < 0.05/**: p value < 0.01/***: p value < 0.001).

these mice was died before completion of the test.), and in the control group, 83.3% (6 out of 7 mice).

5. Discussion

As *Leishmania major* is an intracellular parasite, control of zoonotic cutaneous leishmaniasis mainly relies on cellular immunity.[24,25] Huge number of vaccine strategies have been trailed including the use of live vaccines (leishmanization), killed vaccines, Recombinant proteins, novel vaccine strategies and genetically modified parasites (DNA vaccines).[26–28] Also, different adjuvants have been used for developing an effective vaccine against *L. major* such as Granulocyte macrophage-colony stimulating factor (GM-CSF), *Bacille Calmette Guérin* (BCG), Montanide ISA 720, Aluminum salts, Monophosphoryl lipid A, Saponins (Quil-A, ISCOM and QS-21) and Freund's adjuvants.[29] To date, no approved vaccine has been developed for human leishmaniasis,[30] Therefore, selection of antigen and adjuvant has a great importance in vaccine developing studies.[31] In this study, three different *L. major* antigens were utilized (formaline killed promastigotes (FKP), autoclaved *Leishmania major* (ALM) and soluble Leishmania antigen (SLA) which were used in previous studies). Additionally, alum (known adjuvant usable in human vaccines) and naltrexone (it is already used as a medicine in humans) were used as adjuvant because of their good act in stimulating the immune system in our previous studies.[18,32] In present study the aim was a comparison between immunogenicity potential of ALM, FKP and SLA antigens.

There is a close relationship between IgG different isotypes production and T cell-specific cytokines (such as IL-4), so that IgG1 production is dependent on TH2 relative cytokines[33] and TH1 cytokines including IFN-γ influence the production of IgG2a.[34] In our study, serum anti-ALM total IgG, IgG1 and IgG2a antibodies showed a significant increase in comparison with control groups, indicating that Alum-Nalt has induced effectively cellular and humoral immune responses.

Our results showed that, ALM antigen in association with Alum-Nalt adjuvant enhances both cellular and humoral immune responses including splenic lymphocyte proliferation and production of IFN-γ (the major cytokine in cellular response against *L. major* infections) and IL-5 cytokines (one of TH2 cytochines). Enhanced levels of Th2 mediated factors including splenic IL-5 and serum ALM-specific IgG1 in adjuvant groups in comparison with adjuvant free groups, have confirmed the potential role of alum in shifting the immune responses to humoral immune profile and increased levels of cellular immune mediated factors including splenic lymphocyte proliferation responses, splenic IFN-γ and serum ALM-specific IgG2a in mixture adjuvant receiving groups in comparison with adjuvant free groups, have confirmed the potential role of naltrexone in shifting the immune responses to Th1 profile. Furthermore it was understood that Alum had a synergistic effect on naltrexone by an increased antigen presenting activities. Comparison between the results of adjuvant and adjuvant free groups, showed the adjuvant mixture ability in increasing immune activities at both Th1 and Th2 cells against the antigen.

As mentioned above, the most effective immune response against many pathogens, even for some obligate intracellular pathogens, is an immune response that combines both humoral and cellular components. Furthermore it was understood that the

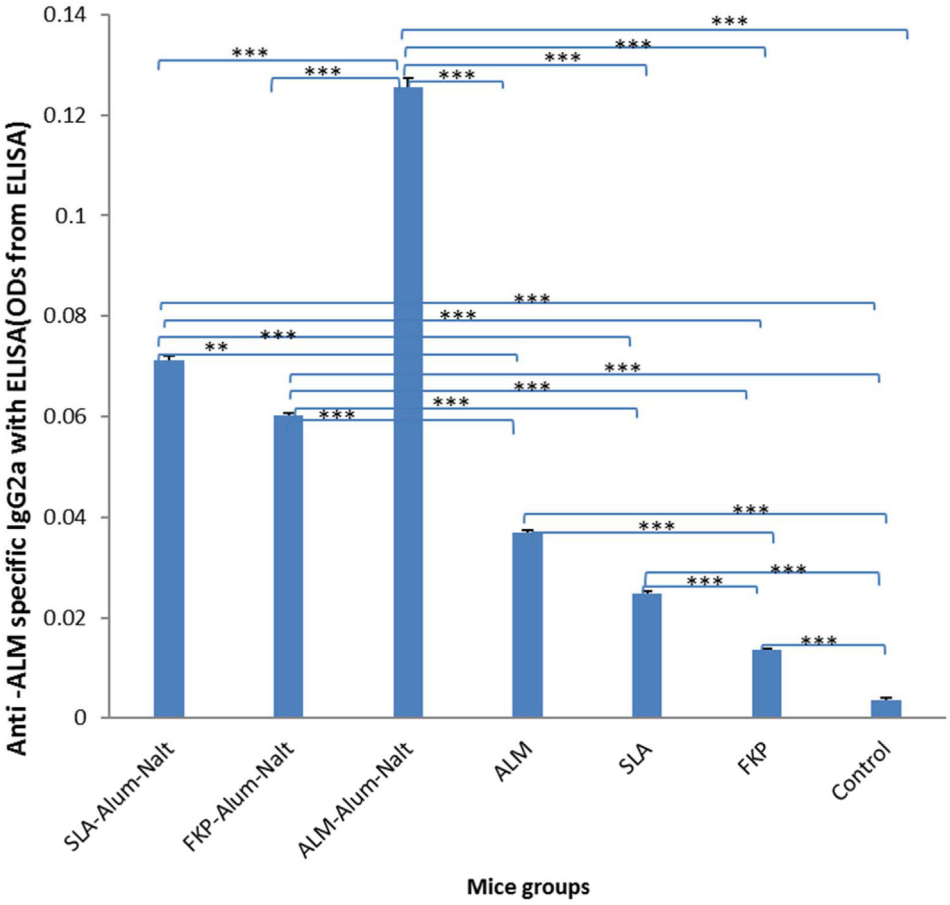

Fig. 6. Comparison of optical densities from ELISA test for serum anti-ALM-specific IgG_{2a} among different groups of mice. (*: p value < 0.05/**: p value < 0.01/***: p value < 0.001).

mechanism of naltrexone action is to provide a pro-inflammatory milieu by blocking opioid receptors or to trigger a low-grade inflammation by increasing the release of local para-inflammatory neuropeptides. Alum had a synergistic effect on naltrexone by an increased antigen presenting activities and alum can indirectly increase the expression of neurokinins on local innate immune cells.[35] The alum mediated increase in the expression of neurokinins may increase the effects of naltrexone induced proinflammatory neuropeptides on innate immune cells. Also, according to our results, adjuvant receiving mice groups, have lower lesion formation in comparison with adjuvant free groups and no wound found in ALM-Alum-Nalt group that indicates the high efficacy of ALM antigen and Alum-Nalt adjuvant in comparison with FKP and SLA antigens.

6. Conclusions

According to the results from this study, the combination of ALM antigen of *L. major* and Alum-Nalt adjuvant is capable to activate cellular immunity and prevent formation of ulcers, but for the next studies we recommend a greater number of cases to be used for challenge test.

Ethical issues

The study was approved by Ethics Committee of Urmia University of Medical Sciences.

Conflict of interest statement

None.

Acknowledgments

This study was financially supported by Urmia University of Medical Sciences, Urmia, Iran (1704). The authors wish to thank Dr. Sh. Khademvatan for his assistance and Urmia University of Medical Sciences for financing the project.

References

1. Bessat M, Okpanma A, Shanat E. Leishmaniasis: epidemiology, control and future perspectives with special emphasis on. *Egypt J Trop Dis*. 2015;2.
2. Handman E. Leishmaniasis: current status of vaccine development. *Clin Microbiol Rev*. 2001;14:229–243.
3. Matlashewski G. Leishmania infection and virulence. *Med Microbiol Immunol*. 2001;190:37–42.
4. Salam N, Al-Shaqha WM, Azzi A. Leishmaniasis in the Middle East: incidence and epidemiology. *PLoS NeglTrop Dis*. 2014;8:e3208.
5. Kedzierski L. Leishmaniasis vaccine: where are we today? *J Global Infect Dis*. 2010;2:177.
6. Engers H, Bergquist R, Modabber F. Progress on vaccines against parasites. *Dev Biol Stand*. 1995;87:73–84.
7. Sadick MD, Locksley RM, Raff HV. Development of cellular immunity in cutaneous leishmaniasis due to Leishmania tropica. *J Infect Dis*. 1984;150:135–138.
8. Aguilar J, Rodriguez E. Vaccine adjuvants revisited. *Vaccine*. 2007;25:3752–3762.
9. Sohrabi Y, Jaafari MR, Mohammadi A, Eskandari S, Khamesipour A. Evaluation of immune response against leishmaniasis in resistance C57 BL/6 mice

immunized with liposomes containing autoclaved Leishmania major with BCG. *Cell Mol Biol Lett*. 2005;10(Suppl 1):S98.
10. Reed SG, Bertholet S, Coler RN, Friede M. New horizons in adjuvants for vaccine development. *Trends Immunol*. 2009;30:23–32.
11. Walker DM, Oghumu S, Gupta G, McGwire BS, Drew ME, Satoskar AR. Mechanisms of cellular invasion by intracellular parasites. *Cell Mol Life Sci*. 2014;71:1245–1263.
12. De Gregorio E, Tritto E, Rappuoli R. Alum adjuvanticity: unraveling a century old mystery. *Eur J Immunol*. 2008;38:2068–2071.
13. Gupta RK, Rost BE, Relyveld E, Siber GR. *Adjuvant properties of aluminum and calcium compounds. Vaccine design*. Springer; 1995. 229–248.
14. Vo HTM, Baudner BC, Sammicheli S, Iannacone M, D'Oro U, Piccioli D. alum/Toll-like receptor 7 adjuvant enhances the expansion of Memory B cell compartment Within the Draining lymph node. *Front Immunol*. 2018;9:641.
15. Khorshidvand Z, Shahabi S, Mohamadzade H, Daryani A, Tappeh KH. Mixture of alum–naloxone and alum–naltrexone as a novel adjuvant elicits immune responses for Toxoplasma gondii lysate antigen in BALB/c mice. *Exp Parasitol*. 2016;162:28–34.
16. Law P-Y, Wong YH, Loh HH. Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol*. 2000;40(1):389–430.
17. Burris S, Norland J, Edlin BR. Legal aspects of providing naloxone to heroin users in the United States. *Int J Drug Policy*. 2001;12(3):237–248.
18. Shahabi S, Azizi H, Mazloomi E, Tappeh KH, Seyedi S, Mohammadzadeh H. A novel adjuvant, the mixture of alum and naltrexone, augments vaccine-induced immunity against Plasmodium berghei. *Immunol Invest*. 2014;43(7):653–666.
19. Momeni AZ, Jalayer T, Emamjomeh M, et al.. A randomised, double-blind, controlled trial of a killed L. major vaccine plus BCG against zoonotic cutaneous leishmaniasis in Iran. *Vaccine*. 1999;17(5):466–472.
20. Mutiso JM, Macharia JC, Mutisya RM, Taracha E. Subcutaneous immunization against Leishmania major-infection in mice: efficacy of formalin-killed promastigotes combined with adjuvants. *Revista do Instituto de Medicina Tropical de São Paulo.*. 2010;52(2):95–100.
21. Ul Bari A. Epidemiology of cutaneous leishmaniasis. *J Pakistan Assoc Dermatol*. 2006;16:156–162.
22. Jazani NH, Karimzad M, Mazloomi E, et al.. Evaluation of the adjuvant activity of naloxone, an opioid receptor antagonist, in combination with heat-killed Listeria monocytogenes vaccine. *Microbes Infect*. 2010;12(5):382–388.
23. Jamali A, Mahdavi M, Shahabi S, et al.. Naloxone, an opioid receptor antagonist, enhances induction of protective immunity against HSV-1 infection in BALB/c mice. *Microb Pathog*. 2007;43(5):217–223.
24. Awasthi A, Mathur RK, Saha B. Immune response to Leishmania infection. *Indian J Med Res*. 2004;119(6):238.
25. Gicheru M, Mutiso J, Macharia J, Ozwara H. *Immunol Leishmaniasis*. 2013.
26. Dunning N. Bioscience Horizons Advance Access published February 17, 2009; 2009.
27. Mendonça SC. Differences in immune responses against Leishmania induced by infection and by immunization with killed parasite antigen: implications for vaccine discovery. *Parasites Vectors*. 2016;9(1):492.
28. Kumar R, Engwerda C. Vaccines to prevent leishmaniasis. *Clin Transl Immunol*. 2014;3(3):e13.
29. Mutiso JM, Macharia JC, Gicheru MM. A review of adjuvants for Leishmania vaccine candidates. *J Biomed Res*. 2010;24(1):16–25.
30. Srivastava S, Shankar P, Mishra J, Singh S. Possibilities and challenges for developing a successful vaccine for leishmaniasis. *Parasites Vectors*. 2016;9(1):277.
31. Kenney RT, Sacks DL, Sypek JP, Vilela L, Gam AA, Evans-Davis K. Protective immunity using recombinant human IL-12 and alum as adjuvants in a primate model of cutaneous leishmaniasis. *J Immunol*. 1999;163(8):4481–4488.
32. Hajipirloo HM, Bozorgomd A, Shahabi S, Tappeh KH, Karamati SA. Evaluation of alum-naltrexone adjuvant activity, on efficacy of anti-leishmania immunization with autoclaved leishmania major (MRHO/IR/75/ER) antigens in BALB/C mice. *Iran J Parasitol*. 2014;9(3):311.
33. Fallon PG, Jolin HE, Smith P, et al.. IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity*. 2002;17(1):7–17.
34. Snapper CM, Peschel C, Paul WE. IFN-gamma stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. *J Immunol*. 1988;140(7):2121–2127.
35. Jazani NH, Sohrabpour M, Mazloomi E, Shahabi S. A novel adjuvant, a mixture of alum and the general opioid antagonist naloxone, elicits both humoral and cellular immune responses for heat-killed Salmonella typhimurium vaccine. *FEMS Immunol Med Microbiol*. 2011;61(1):54–62.