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### Original Article

## 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

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#### **Abstract**

**Background:** Naltrexone, an opioid receptor antagonist shifts the immune response toward a Th1 profile. In the current study, we evaluated the efficacy of the mixture of NTX and alum, as a new adjuvant, to enhance immune response and induce protection against *Leishmania major* in a mouse model.

**Methods:** BALB/c mice were immunized three times either autoclaved *L. major* promastigotes' antigens alone or in combination with the adjuvant alum, naltrexone or the alum–naltrexone mixture. Both humoral and cellular immune responses were assessed two weeks after the last immunization and compared with control mice.

**Results:** The administration of alum- NTX in combination with the parasite antigen, significantly increased production of IFN- $\gamma$ , IFN- $\gamma$  /IL-5 ratio, lymphocyte proliferation and improved DTH response against *L. major*. There was no significant difference in survival following challenge among groups.

**Conclusion:** Immunization with the alum– naltrexone mixture as an adjuvant, in combination with the autoclaved *L. major* promastigotes antigens, can enhance cellular immunity and shift the immune responses to a Th1 pattern.

## Introduction

Cutaneous leishmaniasis (CL) is endemic in more than 70 countries in the world (1). The available drugs for leishmaniasis treatment are expensive and toxic, cause severe side effects and there is an increasing incidence of drug resistance (2, 3). Therefore there is a need for an effective vaccine to control and prevent from this disease.

However, in spite of intensive efforts during the past decades, only a few first vaccines generation of whole killed *Leishmania* reached to phase 3 of clinical trials. This type of vaccines have shown poor efficacy (3, 4). It seems that the reason of this failure is mainly due to lack of an appropriate adjuvant (2).

An Inoculation of live virulent parasites known as leishmanization (LZ) was practiced in several countries including Iran and Uzbekistan, Due to safety concerns and difficulties in standardization of the injected parasites, LZ practice was stopped in different countries except in Uzbekistan (5, 6).

A large number of adjuvants and delivery systems like BCG, G-CSF, IL-12, CpG oligonucleotides, PLGA microspheres and liposomes have been used to potentiate the immune response against *Leishmania* antigens in animal models (3, 4, 6-8). *Mycobacterium bovis* - Bacillus Calmette-Guérin (BCG) has been used in field trial as an immunoadjuvant against different forms of leishmaniasis. (8-12). Vaccination with autoclaved *L. major* (ALM) mixed with BCG was found to be safe but did not induce significant protective immune response in healthy volunteers against cutaneous leishmaniasis (11, 12); furthermore in several studies *M. bovis* BCG inoculation induced auto-immune reactions (13).

The only vaccine adjuvant that is approved by FDA is alum (aluminum-based mineral salt) (14). The major limitations of alum is its poor inducer to elicit cell-mediated immunity and T helper 1 (Th1) responses that are required to protect against intracellular patho-

gens (15, 16). Different studies showed that aluminum compounds need immunostimulatory adjuvants to induce protection against leishmaniasis.

Opioids have significant role in the modulation of Th 1/Th 2 balances (17). Exposure to opioid peptides increased susceptibility to microbial infection (18). Naltrexone (NTX), as opioid antagonist, is capable to block  $\mu$ -opioid receptors and reduces the positive reinforcing effects of opioids (19, 20). Naltrexone can enhance lymphocyte proliferation and shift the immune response toward a Th1 pattern (21). Furthermore, that NTX reduce viral replication and inhibit tumor growth (19, 22, 23). NTX is a long-acting opioid receptor antagonist that is approved by the FDA as a prescription drug and is widely used for treating alcohol and opiate addiction (20). In the current study, we tested the immunogenicity potential of naltrexone alone or in mixture with alum as an adjuvant against heat-killed *L. major* promastigotes in the susceptible BALB/c mice.

## Materials and Methods

Female BALB/c mice (6–8 weeks old) were purchased from the Razi Vaccine and Serum Research Institute of Iran. Experiments were performed in accordance with the Animal Care and Use Protocol of Urmia University of Medical Sciences.

### *Parasites and sample preparation.*

The *L. major* strain MRHO/IR/75/ER was provided from School of Public Health, University of Tehran. Promastigotes of *L. major* were harvested from stationary growth phase by centrifugation (2000 g, 20 min, 4°C), washed 3 times with cold PBS (pH 7.2) and homogenized with glass-glass homogenizer followed by autoclaving. Total protein content of the autoclaved antigen was determined by Bradford method (each dose containing 40  $\mu$ g/mL of

protein parasite). Antigen suspension was kept in small aliquots at  $-20^{\circ}\text{C}$  until use.

### ***Immunization of BALB/c mice***

Female BALB/c mice were divided into five groups (10 mice per group). Each group was divided to two subgroups, five mice in each subgroup: one subgroup was evaluated for lymphocyte proliferation and shift the immune response to Th1/Th2 and the second subgroup was assessed for Delayed Type Hypersensitivity (DTH) and challenge by the parasite. The alum-NTX mixture was prepared by thoroughly mixing 50  $\mu\text{l}$  of PBS containing neltrexone (Sigma, Germany) at a concentration of 6 mg/kg with 50  $\mu\text{l}$  of alum (aluminum phosphate, Sigma). This mixture was incubated in sterile conditions for 72 h at  $4^{\circ}\text{C}$ . BALB/c mice were immunized subcutaneously three times at 2-week intervals with one of the followings: Group Vac (50  $\mu\text{l}$  Ag + 100  $\mu\text{l}$  PBS /mouse), Group Al-Vac (50  $\mu\text{l}$  Ag + 50  $\mu\text{l}$  Al + 50  $\mu\text{l}$  PBS /mouse), Group NTX-Vac (50  $\mu\text{l}$  Ag + 50  $\mu\text{l}$  NTX + 50  $\mu\text{l}$  PBS /mouse), Group AL-NTX-Vac (50  $\mu\text{l}$  Ag + 50  $\mu\text{l}$  Al + 50  $\mu\text{l}$  NTX /mouse) and Control mice received 150  $\mu\text{l}$  of PBS.

### ***Lymphocyte proliferation test***

Two weeks after the last immunization, the spleens were aseptically removed (five mice from each group) and separately homogenized in incomplete RPMI 1640 (Gibco-BRL). Erythrocytes were lysed with ammonium chloride (0.9%) and the splenocytes were washed three times with incomplete RPMI 1640. The cell concentration was adjusted to  $1 \times 10^6$  cells/ml in complete RPMI (CCM) containing 10% FBS (Gibco-BRL), 2mM L-glutamine, 100  $\mu\text{g}/\text{ml}$  streptomycin and 100 IU/ml penicillin. One hundred microliters of diluted cell suspensions were dispensed into 96-well flat-bottom culture plates. Five  $\mu\text{l}$  of the antigen suspension was added to each well and the volume was adjusted to 200  $\mu\text{l}$  with CCM. Control wells were made with 100  $\mu\text{l}$  of diluted cell suspension from the same mouse

and 100  $\mu\text{l}$  of CCM. Each mouse's splenocytes were plated in duplicate. Lymphocyte proliferation was measured by an MTT assay (Thiazolyl Blue Tetrazolium Bromide, Sigma, Germany) after 48 hours' incubation in  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ .

### ***Cytokines analysis***

Spleen cells were removed and cultured as above and after 72 h of culture the levels of cytokines IFN- $\gamma$  and IL-5 in the culture supernatant were measured by commercial kit (ELISA PRO kit for Mouse IFN- $\gamma$ , IL-5, MabTech).

### ***Delayed type hypersensitivity (DTH) assessment***

*Leishmania major* promastigotes were collected at the stationary phase by centrifugation, washed three times with cold PBS and diluted with PBS to have  $1 \times 10^7$  promastigotes per ml. The cells were lysed by six cycles of freeze thawing and stored at  $-20^{\circ}\text{C}$ . Three weeks after the last immunization, DTH was assessed by SC injection of 50  $\mu\text{l}$  from this promastigote crude lysate into left footpad. As a control, the right footpad was injected with 50  $\mu\text{L}$  of PBS alone. The difference in footpad swelling was measured at 72 hours using a metric caliper.

### ***Challenge with live promastigotes of L. major***

Four weeks after last immunization, all groups (5 mice per group) were challenged subcutaneously in the tail base of the mice with  $1 \times 10^6$  stationary phase promastigotes. Lesion development and survival rates of the vaccinated and control mice were weekly monitored after the parasite challenge.

### ***Statistical analysis***

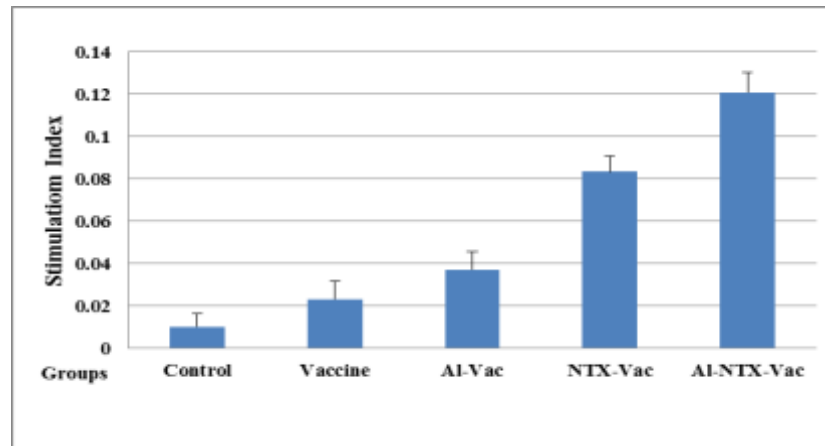
All experiments were analyzed by variance Analysis (ANOVA) followed by Tukey test.  $P < 0.05$  was considered statistically significant.

## Results

### Lymphocyte proliferation

As it is shown in Fig. 1, the mean level of MTT in the mice immunized with Al- NTX – Vac and NTX – Vac induced significantly higher lymphocyte proliferation comparing to the control group ( $P < 0.001$  and  $P < 0.01$  respectively). Spleen cell proliferation with Al-

NTX– Vac ( $P < 0.001$ ) was significantly higher than Al- Vac and Vac groups. Furthermore lymphocyte proliferation was significantly higher in mice immunized with the NTX – Vac compared with mice that received antigen alone ( $P < 0.05$ ). There was no significant difference in lymphocyte proliferation between other groups.



**Fig. 1:** Lymphocyte proliferation response to the Al- NTX 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. The highest stimulation index was observed in the splenocytes of the Al- NTX – Vac immunized mice. Stimulation indices (SI) were determined and expressed as differences between the absorbance of treated and untreated wells. The values are mean  $\pm$  SE (n= 5 mice per group)

### In vitro cytokine production by splenocytes

As it is shown in Fig. 2, the supernatant of splenocytes of mice immunized with Al- NTX– Vac or NTX– Vac showed significantly the highest ( $P < 0.01$ ) level of IFN- $\gamma$  compared with the other groups. There was no significant difference in level of IFN- $\gamma$  between other groups.

The mice of NTX- Vac group produced the highest amounts of IL-5 but IFN- $\gamma$  to IL-5 ratio was higher in Al-NTX- Vac group, none of them proofed as significant (Table 1).

### Delayed type hypersensitivity responses (DTH)

To assess cell-mediated responses to parasite antigens *in vivo*, the DTH reaction was meas-

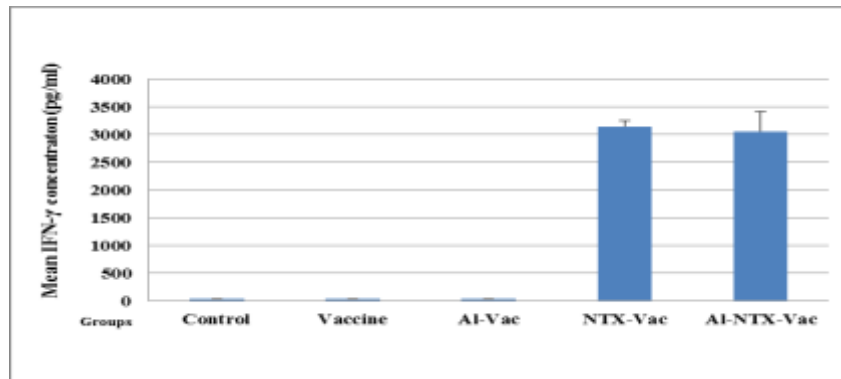
ured 72 h post infection. The results of DTH showed that different groups of immunized mice induced a stronger DTH response than PBS control group (Fig. 3); however, the difference was not statistically significant except for the Al- NTX – Vac group ( $P < 0.01$ ).

### Challenge results

Stationary phase promastigotes of *L. major* were injected subcutaneously at the tail base of 5 mice from each group, an injection site was analyzed over a period of 10 weeks. There was no significant difference between immunized and control groups of mice, during 10 weeks after challenge with *L. major* (data not shown).

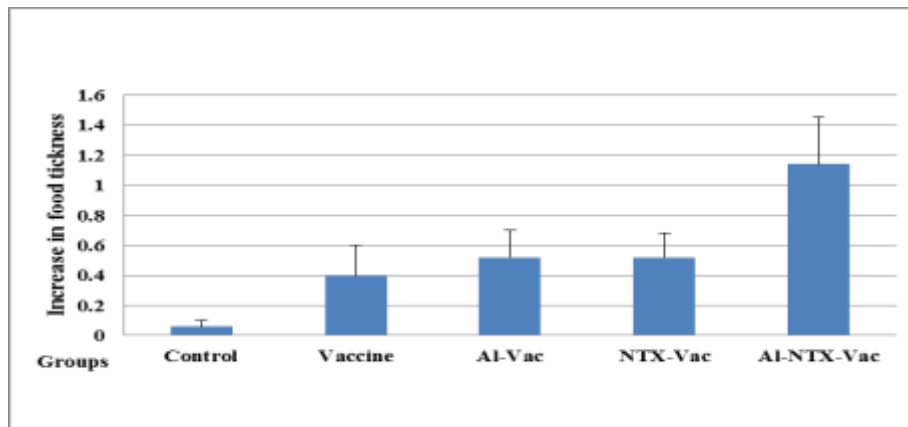
**Table 1:** Levels of IFN- $\gamma$  and IL-5 cytokines in spleen cells immunized mice after in vitro stimulation with parasite antigen. Results indicate that co-administration of Al- NTX as adjuvant induced higher IFN- $\gamma$  /IL-5 ratio against antigens *Leishmania* in spleen cells of BALB/c mice

Immunization Groups	IFN- $\gamma$	IL-5	IFN- $\gamma$ /IL-5
PBS	35.65784	0.803187	131.0214
Vaccine	35.43938	2.407359	829.9403
Al- Vac	35.49241	10.56552	20.27474
NTX- Vac	3143.41	15.96009	345.4332
Al- NTX- Vac	3061.204	9.038719	4855.173



**Fig. 2:** IFN- $\gamma$  production by splenocytes of the immunized mice

Two weeks after last immunization the level of IFN- $\gamma$  was measured by ELISA in the supernatants of cells cultures from five mice per group. Cells were stimulated in vitro for 72h with 5  $\mu$ g/ml of parasite antigen. The level of IFN- $\gamma$  in mice immunized with Al- NTX and NTX higher than the other groups. The values are mean  $\pm$ SE (n= 5 mice per group)



**Fig. 3:** DTH response to parasite antigens in BALB/c mice immunized with different adjuvants

Three weeks after last immunization  $1 \times 10^7$  freeze-thawed *L. major* promastigotes and PBS in a final volume of 50  $\mu$ l was injected in left and right footpad of mice respectively. The difference in footpad swelling was measured at 72 hours using a metric caliper. Result showed that there was a significant difference ( $P < 0.01$ ) in induction of DTH response Al- NTX -Vac as compared to the control group of mice. No significant difference was seen between other groups

## Discussion

In this study, alum naltrexone as a Th1 immunostimulatory adjuvant has been used for the first time with autoclaved *L. major* to elicit a protective immune response challenge with the parasite in susceptible BALB/c mice. Our selection to test naltrexone in this paper was based on reports of the literature indicating that has been effective in shifting immune responses to some antigens from a Th2 response to Th1 immune responses (21-23).

Brown et al. suggested that low-dose naltrexone presents a safe and promising approach to prevention and/or treatment of many autoimmune diseases and cancer variants, as well as potentially various viral (AIDS) and neurological diseases (Multiple Sclerosis) that are exacerbated by compromised immunity (22).

Moreover, naltrexone was reported to stimulate IFN- $\gamma$  production and induced effective immune responses against fibrosarcoma tumor and led to a significant inhibition of tumor growth in BALB/c mice (23). The finding of the current study about adjuvant activity of naltrexone and previous studies about adjuvant activity of naloxone (which are structurally similar pure opiate receptor antagonists) and propranolol (24-28), emphasizes that the local microenvironment at the time of up taking and processing an antigen by APCs has a key role in the fate of subsequent acquired immune response against infection (29, 30). One important mechanism for adjuvant activity of naltrexone is blocking the  $\mu$  opioid receptors which accelerates local inflammation via direct effect on monocytes, macrophages and dendritic cells (31, 32).

IFN- $\gamma$  secreted by T cells, plays a key role in protection against intracellular infectious agents and is the cytokine primarily used as a marker for the existence of Th1 immune responses. Cytokine analysis revealed that the administration of *Leishmania* proteins with Al-NTX or NTX induced high levels of Th1

cytokine (IFN- $\gamma$ ). Furthermore, the spleens of mice immunized with Al-NTX-Vac showed significantly the highest IFN- $\gamma$  /IL-5 ratio compared with the other immunized groups which is an important correlate of immune protection against *L. major*. These results are in agreement with other studies showing that NTX induce Th1 type of immune response (23, 32). NTX can shift the immune response toward a Th1 pattern while alum is a Th2-type adjuvant. However, despite the opposing effects of these adjuvants on skew the immune system toward a TH1/TH2 response, our results show that co-administration of naltrexone with alum more skewed the immune response in BALB/c mice towards a Th1-type than administration of naltrexone alone.

Evaluation of lymphocyte proliferation response revealed that immunization of mice with Al-NTX-Vac stimulate the proliferation of spleen cells significantly moreover footpad swelling after infectious with *L. major* was measured and the results demonstrated that Al-NTX-Vac elicited strong DTH responses.

Unexpectedly, immunization of BALB/c mice with different groups and challenging with *L. major* did not show any significant differences in the death rate until 10 weeks after infection.

## Conclusion

Immunization with the alum- naltrexone mixture as an adjuvant, in combination with the autoclaved *L. major* promastigotes antigens, can enhance cellular immunity and shift the immune responses to a Th1 pattern. To our awareness, this study is the first evaluation for using an alum- naltrexone mixture as an adjuvant for vaccination. Therefore, follow-up studies are needed to elucidate the effect of naltrexone on the mice immune system and to examine adjuvant activity of naltrexone when combined with vaccines against other microorganisms.

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