Full length article

Mixture of Alum − Naloxone and Alum − Naltrexone as a novel adjuvant elicits immune responses for *Toxoplasma gondii* lysate antigen in BALB /c mice

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HIGHLIGHTS

- Alum-Naltrexone induced high level of cellular immune response.
- Alum-Naltrexone induced lymphocyte proliferative response and Th1-type immune response significantly.
- Alum improved the conditions for the Naltrexone and Naloxone.
- Immunization with Alum-Naltrexone prolonged survival time in mice.

GRAPHICAL ABSTRACT
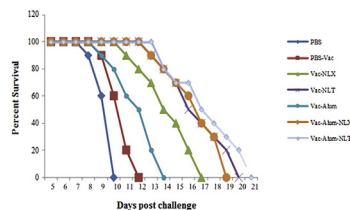
Vac-Alum-NLT: Aluminum phosphate-Naltrexone), Vac-Alum-NLX: Aluminum phosphate-Naloxone, Vac-NLT: vaccine Naltrexone, Vac-Alum: vaccine Aluminum phosphate, Vac-NLX: vaccine Naloxone, TLA: *T. gondii* lysate antigen.

ARTICLE INFO

ABSTRACT

Toxoplasma gondii (*T. gondii*) is an obligate intracellular parasite. Treatment of the infection induced by this parasite is not straightforward due to the toxic side effects of the available drugs. Vaccine development could be a solution to this problem. In the present study, *T.gondii* Lysate Antigen (TLA), as a model vaccine, in combination with the Alum-NLT (Aluminum phosphate-Naltrexone) and Alum-NLX (Aluminum phosphate-Naloxone) were evaluated for immunization BALB/c. 147female BALB/c mice which were divided into seven groups of 21, were allocated to immunization experiments. The first group was selected as the negative control group, followed by the second, third, fourth, fifth, sixth and seventh groups which were immunized with Vac, Vac-Alum, Vac-NLX, Vac-NLT, Vac-Alum-NLX, Vac-Alum-NLT, respectively. Ten days after the final immunization, mice in all groups were divided into three groups for evaluating cellular immune responses, measuring the delayed-type hypersensitivity responses (DTHs) and evaluating survival. The DTH and cellular immune responses showed that in mice immunized with the TLA vaccine combined with the Alum-NLT mixture, the efficacy improved by increasing the production of Interleukin-5(IL-5) and Interferon gamma. This consequently shifted the immune responses toward a Th1 profile by increasing the IFN-γ/IL-5 ratios. In challenge experiments, immunized mice with the Alum-NLT-Vac mixture survived for a longer period of time which indicated an improvement in protective immunity against *T. gondii*. Administration of the Alum-NLT mixture

adjuvant in combination with TLA vaccine enhanced the cellular immunity by shifting the immune response to a Th1 pattern. This shift to the Th_1 pattern plays an important role in the induction of cellular.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite of warm-blooded animals. It is a coccidian parasite with cats as the definitive host, and warm-blooded animals as intermediate (Frenkel, 1970). The parasite is distributed among humans and mammals worldwide (Kuk and Ozden, 2007). This protozoan can invade a wide range of cells. The host cell invasion is a crucial step in inducing the infection. Inside the host cells most apicomplexan parasites reside and develop within a vacuole termed the parasitophorous vacuole (PV) (Mordue et al., 1972). Although, *T. gondii* most often causes subclinical infection, primary infection during pregnancy can induce fetal pathology and absorption in both humans and lower animals. In the chronic phase, in immuno-compromised patients (such as in AIDS patients), the reactivation of encysted bradyzoites into the actively replicating and cytolytic tachyzoites can lead to fatal toxoplasmic encephalitis (TE), pneumonitis and myocarditis (Barbosa et al., 2007).

Protection against intracellular parasite requires T-helper 1 (Th1)-oriental immune responses, mainly by an IFN-γ driven Cytotoxic T lymphocytes (CTL) response. Infection with *T. gondii* induces the proinflammatory cytokines, such as Tumor Necrosis Factor-α (TNF-α), IL-12 and IFN-γ, in macrophages and lymphocytes, which are essential to control both chronic and acute phases of *T. gondii* infection (Denkers and Gazzinelli, 1998). IFN-γ is a key player in limiting parasitic growth (Suzuki et al., 1988). Treatment of *T. gondii*-induced infection is an obstacle due to the toxic effects of the available drugs, as well as the immediate reoccurrence of infection in immuno-compromised patients. In veterinary medicine, *T. gondii* infection is a problem for sheep farmers, causing considerable economic loss or as a source of transmission to humans (Meerburg et al., 2006). Therefore, vaccine development against *T. gondii* infection may contribute to alleviating these problems. The efficacy of a vaccine depends on some parameters, including immune-stimulatory properties of adjuvants applied and delivery system for both antigen and adjuvant. Evidence indicates that adjuvant plays an important role in the efficacy of immunization (Zimmermann et al., 2008; Jongert et al., 2009).

Aluminum compound is the only vaccine adjuvant that is approved by the United States Food and Drug Administration (FDA) and has been used as a human vaccine adjuvant for more than 70 years. Previous studies have shown that the immune adjuvant effect of Alum could play an important role in conferring immunity, by inducing a quick increased production of systemic IFN-γ in host (Hem and White, 1995; Mazloomi et al., 2012).

Naloxone (NLX) is a general opioid receptor antagonist which is currently approved by the FDA. It is a prescription drug and routinely used for the safely revers opioid-induced respiratory depression (Anon., 2008). This adjuvant can shift the immune response toward a Th1 pattern (Sacerdote et al., 2000). Previous studies have shown that NLX administration as an adjuvant vaccine increases the efficacy of the respective vaccine by enhancing the cellular immunity (Jamali et al., 2007; Jazani et al., 2010). Additionally, NLX as an adjuvant vaccine also skews the immune response toward a Th1 pattern and improves the protective immunity (Jazani et al., 2010).

Naltrexone (NLT), an opioid antagonist, is a synthetic drug. It can occupy the opiate receptors but not the active receptors. NLT has approved by the FDA to be used as a treatment of heroin addiction and alcoholism. Nausea or vomiting can occur in patients who actively use Naltrexone.

The current study was designed to evaluate the potential effect of the mixture of Alum and NLX, Alum and NLT as an adjuvant in *T. gondii* Lysate Antigen (TLA) to induce a Th1 response and its efficiency to protect mice against the highly virulent RH strain *T. gondii*.

2. Materials and methods

2.1. Animals

Inbred BALB/c mice were purchased from Razi Institute of Iran. All mice were female, six to eight weeks old, and documented to be specific-pathogen-free and had free access to food and water. All experiments were conducted following the protocol approved by the Institutional Animal Care and Use at the Urmia University of Medical Sciences (Urmia-Iran).

2.2. Parasite

The *T. gondii* strain RH was used in this study. *T. gondii* RH strain was maintained in our laboratory by intraperitoneal passage in BALB/c mice. Tachyzoites were harvested from the peritoneal cavity of the infected mice, three days post infection, by injecting 1 ml of phosphate buffered saline (PBS), pH 7.2. The peritoneal exudates were passed 10 times through a 27 gage needle to release the intracellular tachyzoites. The exudates were separated using low speed centrifugation (100 g for 5 min at 4 °C) to remove the cellular debris. The parasites washed twice in RPMI-1640 medium (Sigma, Germany) containing 100 IU/ml penicillin and 100 μg/ml streptomycin. A Neubauer chamber at 400× magnification was used to determine the concentration of the tachyzoites (Araujo and Remington, 1974).

2.3. Preparation of TLA

About 2×10^9 of the obtained tachyzoites of the RH strain were washed with PBS and centrifuged three times at 750 g for 15 min at 4 °C. The pellet was solubilized by adding distilled water, and was supplemented with protease inhibitor, 5 mM phenylmethyl sulphonyl fluoride (PMSF). The parasites were disrupted by five times freeze-thawing and the extract was clarified by centrifugation at 2500 g for 15 min at room temperature (RT). The supernatant was filtered using a 0.22 μm Millipore membrane filter. The protein concentration was measured using Bradford method and stored at −80 °C until use (Asia et al., 1987; Daryani et al., 2003).

2.4. Immunizations

For immunization experiments, 147 BALB/c mice (6−8 week-old) were selected and divided into seven groups of 21. The mice were vaccinated with TLA alone or in combination with the adjuvants [50 μl Alum (Aluminum phosphate gel, Sigma, Germany), 6 mg/kg NLX (Sigma, Germany) and 0.5 mg/kg NLT (Sigma, Germany) or the Alum-NLX mixture, Alum-NLT]. Each mouse received 50 μl of TLA containing 20 μg/ml of protein mixed (1:1) with

adjuvant. The first group was selected as the negative control group (non-immunized) and received 150 μl PBS. All mice were immunized three times on days 0, 10 and 20 with a total volume of 150 μl.

2.5. Lymphocyte proliferation assay

Ten days after the final immunization, five mice were selected of the each group (n = 35) for the lymphocyte proliferation assay. The spleens of the mice were harvested under sterile conditions and homogenized in RPMI 1640 medium (phenol red-free). The suspensions were centrifuged at 2000 g for 5 min. Erythrocytes were lysed using 0.75% Tris-NH$_4$CL [ammonium chloride in Tris buffer (0.02%), pH 7.2]. The splenocytes were washed twice with RPMI 1640 medium. The pellets were solubilized after centrifuging by adding the RPMI 1640 medium, supplemented with 10% fetal bovin serum (FBS), 2 mM L-glutamine and 25 mM HEPES. The viability of the splenocytes was determined using Trypan blue (0.4%, w/v) exclusion. The cells were dispensed in 96-well flat-bottom culture plates at a concentration of 1×10^6 cell/ml in RPMI 1640 medium containing 10% FBS. They were then stimulated with total TLA at a concentration of 5 μg and the volume were adjusted to 200 μl per well. Mitogen phytohemagglutinin-A (Gibco-BRL) was used at a final concentration of 5 μg/ml in the positive control group. Cells were incubated for 48 h at 37 °C in a 5% CO$_2$ atmosphere. Cell viability were determined after 48 h incubation using an MTT assay (3[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrezolium bromide) (Sigma, Germany). Briefly, 20 μl of MTT solution (Sigma, Germany) was added to each well and the plates were incubated at 37 °C for 4 h. The supernatant was then aspirated from each well and Pellet was solubilized by adding 100 μl of DMSO (dimethyl sulfoxide). Absorbance was measured at 540 nm in an ELISA reader (Jazani et al., 2010).

2.6. Cytokine assays

Ten days after the last immunization, the spleen of each mouse was removed aseptically and homogenized in RPMI 1640 medium (Sigma, Germany) supplemented with 10% FBS and antibiotics. Red blood cells (RBCs) were then osmotically lysed using ammonium chloride buffer (NH$_4$Cl 0.16 M, Tris 0.17 M). The cells were washed twice with RPMI 1640 medium. Cells were then counted and the viability was determined by trypan blue (0.4% w/v) exclusion. A nominal total of 1×10^6 spleen cells were plated in each well of a 24-well plate using RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/mL streptomycin, and 5×10^{-5} M 2-mercaptoethanol. The spleen cells of each mouse were seeded into two wells. The cells were re-stimulated in vitro using 5 μl of TLA. Plates were then incubated for 72 h at 37 °C in 5% CO$_2$. Supernatants were removed 72 h post-stimulation and stored at −70 °C before measuring level of the secreted interferon gamma (IFN-γ) and interleukin (IL)-5. The concentration of IFN-γ and IL-5 in the supernatants was estimated using (ELISA) kit (Bender Med System, Vienna) (Jamali et al., 2007).

2.7. Delayed type hypersensitivity response (DTH)

Ten days after the last immunization, five mice were selected from each group (n = 35) and sensitized subcutaneously (s.c) with 20 μg/ml of TLA suspended in 100 μl of PBS. Each negative control mouse received 100 μl of PBS which was injected into the right footpad and 100 μl PBS containing 20 μg of TLA which was injected to the left footpad area. Footpad erythema was measured after 24 h.The difference in thickness between the footpad received antigen and those injected with PBS was then calculated (Lunden et al., 1993).

2.8. Challenge of immunized mice

BALB/c mice were infected intraperitoneally with 2×10^3 tachyzoites of the T. gondii 10 days after the last immunization. Mortality was monitored daily for a period of 21 days after the challenge with strain RH (Daryani et al., 2003; Lunden et al., 1993; Yap et al., 1998).

2.9. Statistical analysis

The DTH response, MTT assay, and cytokine level measurement data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's posttest. The survival function was estimated by Kaplan−Meier analysis and then a Log Rank test was done. A P-value of less than 0.05 was considered as a statistically significant difference.

3. Results

3.1. Lymphocyte proliferation assay

The lymphocyte proliferation assay was performed in mice immunized with different adjuvants and in non-immunized mice. The lymphocyte proliferation responses are generally considered to be a measure of cell-mediated immunity. T. gondii-specific lymphocyte proliferations were evaluated using MTT dye assay. The result of this test is shown in Fig. 1. The lymphocyte proliferation rate was significantly higher in mice immunized with TLA vaccine in combination with Alum-NLT mixture compared to that in mice that received Vac-NLT, Vac-Alum-NLX, Vac-NLX, Vac-Alum, TLA vaccine and PBS alone. A significant higher proliferation rate was observed in lymphocyte of the mice that were treated with Vac-NLT compared to those received Vac-Alum-NLX, Vac-NLX, Vac-Alum, TLA vaccine and PBS alone. The lymphocyte proliferation rate was significantly higher in mice immunized with Vac-Alum-NLX mixture compared to those which were treated with Vac-NLX, TLA vaccine alone and the control group. Furthermore, the
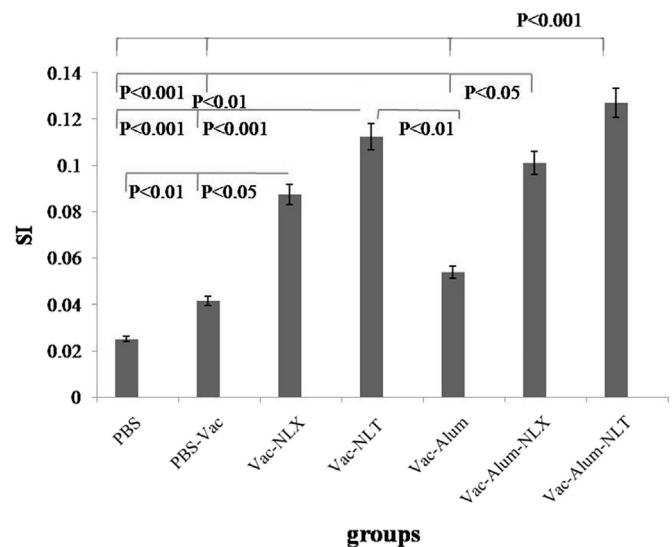
Fig. 1. Effect of administering Alum-NLT and Alum-NLX mixture on lymphocyte proliferation, Ten days after the final immunization, spleen of individual mice (five per group) were removed and lymphocyte proliferation was evaluated dye the MTT method. Values are the mean ± SE. SI. Vac-Alum-NLT: Aluminum phosphate-Naltrexone, Vac-Alum-NLX: Aluminum phosphate-Naloxone, Vac-NLT: vaccine Naltrexone, Vac-Alum: vaccine Aluminum phosphate, Vac-NLX: vaccine Naloxone, Vac-PBS: Vaccine PBS.

lymphocyte proliferation rate was significantly higher in mice received Vac-NLX mixture compared to the mice that were immunized with Vac-Alum, TLA vaccine and PBS alone. Mice that were vaccinated with Alum-Vac mixture had higher level of lymphocyte proliferation compared to the mice treated with TLA vaccine and the negative control mice. Finally, higher level of lymphocyte proliferation was measured in mice that were immunized with TLA vaccine alone in comparison with the mice that only received PBS.

3.2. Cytokine assays

The IFN-γ is defined as a key mediator of responsive immunity during both chronic and acute phases of T. gondii infection in mice. Concentration of IFN-γ and IL-5 in supernatants from the 72 h splenocytes cultures which were stored at $-70\ ^\circ$C was measured using an ELISA kit. The results of ELISA test are presented in Fig. 2. Mice immunized with TLA vaccine in combination with Alum-NLT mixture produced significantly higher amount of IFN-γ compared to the mice vaccinated with Vac-NLT, Vac-Alum-NLX, Vac-NLX, Vac-Alum, TLA vaccine and PBS alone (Fig. 3).

3.3. Delayed type hypersensitivity (DTH) test

Ten days after the last immunization, the sensitization of mice with TLA and injection of antigens to different groups of mice, footpad assay reported at 24 h was shown in Table 1. The mice immunized with Vac-Alum-NLT were significantly higher compared to the group administered with Vac-NLT, Vac-Alum-NLX, Vac-NLX, Vac-Alum, TLA vaccine and PBS alone.
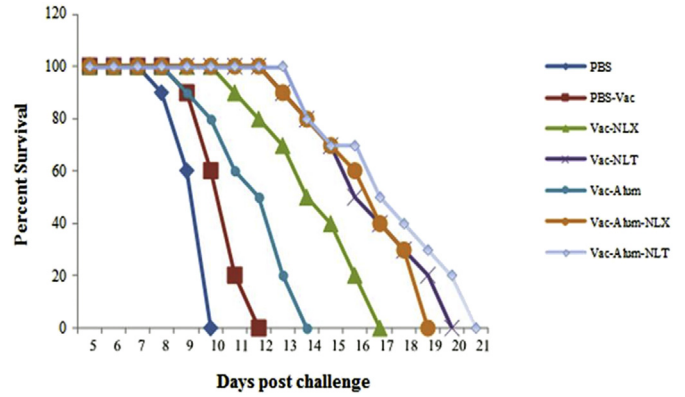
Fig. 3. BALB/c mice were infected intraperitoneally with 2000 tachyzoites of the T. gondii 10 days after the last immunization. Mortality was monitored daily for a period of 21 days after challenge with strain RH. The percentage of survival in the mice immunized with Vac-Alum-NLT, and challenged with RH strain tachyzoites were significantly higher compared to the mice were immunized with the Vac-NLT, Vac-Alum-NLX, Vac-NLX, Vac-Alum, the TLA vaccine, PBS alone. Vac-Alum-NLT: Aluminum phosphate-Naltrexone, Vac-Alum-NLX: Aluminum phosphate-Naloxone, Vac-NLT: vaccine Naltrexone, Vac-Alum: vaccine Aluminum phosphate, Vac-NLX: vaccine Naloxone, TLA: T. gondii lysate antigen.

3.4. Immunization of mice and challenge experiments

Kaplan—Meier curve was plotted for survival in mice (Ten per group) immunized with PBS (control group), Vac, Vac-Alum, Vac-NLX, Vac-NLT, Vac-Alum-NLX and Vac-Alum-NLT and challenged with 2000 tachyzoites of the T. gondii, 10 days after the last
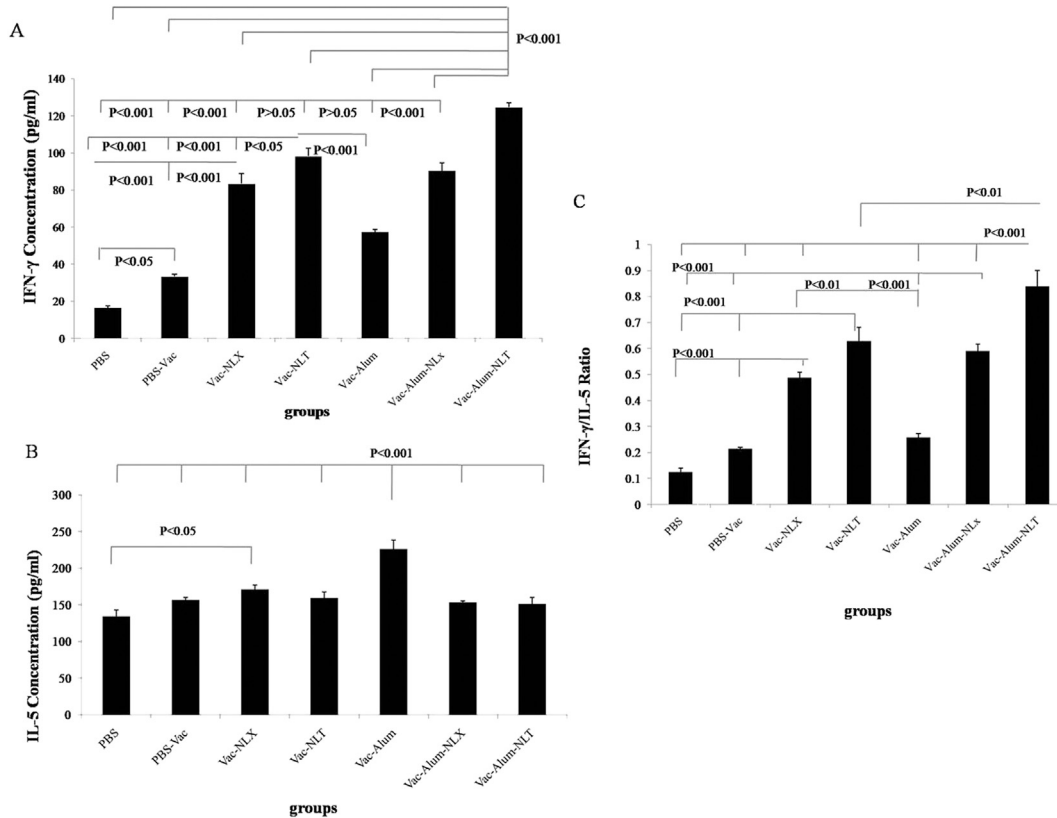
Fig. 2. Effect of administering Alum-NLT and Alum-NLX mixture on IFN-γ and IL-5 production, ten days after the final immunization, spleens of individual mice (five per group) were removed and IFN-γ (A) and IL-5 (B) production and IFN-γ/IL-5 ratio (C). Values are the mean $\pm$ SE. SI. Vac-Alum-NLT: Aluminum phosphate-Naltrexone, Vac-Alum-NLX: Aluminum phosphate-Naloxone, Vac-NLT: vaccine Naltrexone, Vac-Alum: vaccine Aluminum phosphate, Vac-NLX: vaccine Naloxone, Vac-PBS: Vaccine PBS.

Table 1
Mean of the results of DTH reaction in different groups of Balb/c mice examined by footpad.[a] Footpad assay: each data calculated according to the following formula: [(Thickness (in millimeter) of right footpad injected with TLA) − (Thickness of left footpad injected with PBS)] × 100/(Thickness of left footpad injected with PBS).

Groups	Percentage of footpad thickness (mean ± SD) (%)
PBS	3.59 ± 0.57
PBS-Vac	29.1 ± 1.29
Vac-NLX	47.44 ± 3.56
Vac-NLT	53.26 ± 4.643
Vac-Alum	38.7 ± 2.463
Vac-Alum-NLX	51.19 ± 3.1
Vac-Alum-NLT	58.21 ± 4.22

immunization. The mortality rate was checked for 21 days on a daily basis. The survival rate was significantly higher in mice treated with PBS-Vac, Vac-NLX, Vac-NLT, Vac-Alum, Vac-Alum-NLX and Vac-Alum-NLT compared to that in the negative control group ($P < 0.005$). Mice in the Vac-NLX, Vac-NLT, Vac-Alum, Vac-Alum-NLX and Vac- Alum-NLT groups exhibited a significantly higher survival rate compared to those in the PBS-Vac group ($P < 0.02$). The survival rate in mice immunized with Vac-NLX, Vac-NLT, Vac-Alum-NLX and Vac-Alum-NLT was significantly higher compared to those received Vac-Alum ($P < 0.007$). Mice that received Vac-NLT, Vac-Alum-NLX and Vac-Alum-NLT showed a significantly higher survival rate than those in the Vac-NLX group ($P < 0.033$). The survival rate in mice immunized with NLT and Vac-Alum-NLT was higher compared to the Vac-Alum-NLX group and those in the Vac-Alum-NLT group higher than mice in the Vac-NLT group, however; the difference between survival rates was not statistically significant.

4. Discussion

Based on the advances in vaccine adjuvant development in recent years, it seems that adjuvants play an important role in the protective immunity against *T. gondii*. Our finding that the cellular immune responses were stimulated with TLA vaccine in combination with the Alum-NLT mixture strongly supports the use of this combination as an adjuvant. The results of our study showed that Alum-NLT mixture as an adjuvant in combination with the TLA vaccine significantly improved the efficacy of TLA vaccine by increasing the induction of IFN-γ and IL-5 production and shifting the immune responses toward a Th1 profile (by increasing the IFN-γ/IL-5 ratio). Furthermore, the mixture of Alum-NLT as an adjuvant increased the lymphocyte proliferation rate and improved the resistance and survival against a *T. gondii* challenge.

The effects of TLA vaccine in combination with Alum-NLT mixture on cellular immune responses are likely due to Alum and NLT. Several previous studies have confirmed the effectiveness of Alum in immunization against *T. gondii* (Petersen et al., 1998; Dimier-Poisson et al., 2003; Martin et al., 2004) and *Trypanosoma cruzi* (Pereira-Chioccola et al., 1999). The ability of Alum to shift the immune response toward a Th2 profile has been already established (Jazani et al., 2010; Jamali et al., 2009; Harandi et al., 2010). Moreover, it primarily activates IgG$_1$ isotype antibodies (Petersen et al., 1998; Brewer et al., 1996). Although, Alum promotes the production of Th2 cytokines, with a low level of CD8+ T cell activation, converse to the requirements for inducing immunity against *T. gondii* in mice (Zhou et al., 2012). One study demonstrated that specific CTL can be induced by antigen and Alum that can lead to cellular immune responses in mice (Dillon et al., 1992).

Several mechanisms of Alum vaccine-in inducing immune responses, particularly humoral immune, have been explained (De Gregorio et al., 2008; McKee et al., 2009; Wilson-Welder et al.,

2009). It has been reported that Alum can increase the expression of major histocompatibility complex class II and co-stimulatory or adhesion molecules (ICAM-1, LFA3 and CD40) associated with mature dendritic cells (DCs). Alum also enhances the antigen persistence at the injection site and increase recruitment and activation of antigen presenting cells (APCs). Furthermore, stimulation of peripheral blood monocytes with Alum induced an increase in mRNA expression of cytokines, such as IL-1α, IL-β, granulocyte-monocyte colony stimulating factor (GM-CSF) and Tumor Necrosis Factor (TNF) as well as that of IL-4 and IL-6 (Ulanova et al., 2001).

Previous studies have shown that Naloxone (NLX), an opioid antagonist, can shift the immune response toward a Th1 pattern (Sacerdote et al., 2000, 1998). This shift to the Th$_1$ pattern plays an important role in the induction of cellular immunity and increased protection against intracellular parasite. Additionally, it has been previously demonstrated that NLX administration during primary HSV-1 infection with a non-virulent strain significantly increases cellular immunity and IFN-γ production against the virus (Jamali et al., 2007). Three mechanisms by which opioid antagonist may trigger vaccine-induced Th1 and cellular immune responses have already been shown (Jamali et al., 2007; Jazani et al., 2010); **1)** Triggering low grade inflammation by increasing the release of the local pro-inflammatory neuropeptides, such as substance P (SP) which are released by nerve fibers. SP is a potent pro-inflammatory neuropeptide that favors development of cellular immunity (Egan et al., 1998). In such a pro-inflammatory milieu, APCs could polarize specific immune responses toward cell-mediated immunity and shift the immune response to the Th1 pattern (Kaneider et al., 2005; Mathers et al., 2007). **2)** Action is to promote a pro-inflammatory milieu by blocking opioid receptors. This inhibition would accelerate local inflammation via a direct effect on the innate immune cells, for example monocyte, macrophage and dendritic cells. Either of these mechanisms would result in the presentation of TLA antigens by activated APCs (Kirst et al., 2002). **3)** Inhibition of regulatory T lymphocytes. The inhibitory effect of administration of opioid antagonist on regulatory T cells has been shown previously (Molla Hassan et al., 2009). Bayry has already shown that regulatory T lymphocytes can inhibit dendritic cell (DC) maturation and the expression of co-stimulatory molecules which in turn reduce ability of DCs to activate T cells (Houot et al., 2006; Tang et al., 2006; Bayry et al., 2007). NLT-induced inhibition of the interaction of regulatory T lymphocytes with DCs may enhance vaccine-induced immune responses. Therefore, this mechanism is in line with the finding that indicates that the administration of CCR4 antagonist as an adjuvant accelerates vaccine induced immunity by inhibition of regulatory T lymphocytes (Bayry et al., 2008; Davies et al., 2009).

As mentioned above, the action of the Alum-NLT mixture in stimulating cellular immune responses, shifting the immune responses to a Th1 pattern, was more efficacious than stimulation induced by NLT alone. Moreover, the adjuvant activity of the Alum-NLX mixture was more than that of NLX alone. Thus, it seems that Alum augmented the adjuvant activity of NLT and NLX, even in stimulating Th1/cellular responses. This finding is in line with that reported by Su and colleagues indicated that co-administration of Alum and malaria antigen plus IL-12 augmented the ability of IL-12 to induce the production of IFN-γ and high levels of total malaria-specific antibody and IgG2a antibody (Su et al., 2003). It has also been shown that the mice vaccinated with Vac-NLT as an adjuvant have better health condition compared to those that received Vac with NLX. This is probably due to the half-life of NLT in the body that is approximately 4 h and the half-life of NLX which is 45 min (Poole and Peterson, 2005).

5. Conclusion

The present results indicate that administration of Alum-NLT mixture as an adjuvant in combination with TLA vaccine enhanced cellular immunity and shifted the immune response to a Th1 profile. As mentioned above, the adjuvant activities of Alum, NLX, Alum-NLT mixture, and NLT alone were less than that of the Alum-NLT mixture. We recommend that flow-up studies are required to confirm the results of this study and examine the adjuvant activity of the Alum-NLT mixture when combined with vaccines against other *T. gondii* antigens. Success of a vaccine depends on factors, such as the immune stimulatory properties of adjuvants and the delivery systems employed for both antigen and adjuvant.

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