

ORIGINAL ARTICLE

# Studying the Effects of Naloxone-Alum Adjuvant Mixture on Cytokines in Model of Multi-Epitope Vaccine in HIV-1

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## KEYWORDS

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**ABSTRACT:** Cytokines have important roles in the control of bacterial and viral infections such as HIV-1. Interleukin 17 which is secreted by Th17 is one of these cytokines with a special role in controlling microbial infections. In the present study, adjuvant activity of Alum and Naloxone mixture has been studied on immune responses, especially IL-17 cytokine. Naloxone and Alum adjuvant are mixed with 10 µg of recombinant vaccine HIV-1-gag-pol-tat-env. Experimental groups, consisting of 36 inbred male Balb/c mice divided into six groups, were injected subcutaneously at days 0, 14 and 28 with total volume of 200 µl. 2 weeks after final injection, mouse spleens were removed in sterile conditions and used to prepare suspensions. Lymphocyte proliferation responses were evaluated with Brdu test and evaluation of cytokines IL-4, IL-17 and INF-γ were completed using ELISA kit, plus total antibody and antibody isotopes IgG1 and IgG2a using ELISA test. All results show that the mixture of Alum with Naloxone increased cellular immune parameters and specially raised interleukin 17 which illustrated a significant difference with other groups. It seems that Alum and Naloxone mixture could control viral infections by affecting the Th17 pathway in which IL17 cytokine has a critical role.

## INTRODUCTION

Despite extensive efforts in the field of designing HIV vaccines in the world, the development of a safe and effective vaccine still remains elusive [1, 2]. Today, with the addition of adjuvants, the immunogenicity of vaccines has been increased. Adjuvants are generally compounds which do not role of antigenic effects, but

they can be used in stimulating the immune system and increasing immune responses to antigens utilized in designing and producing convenient vaccines [3,6].

One of the most well-known adjuvants used in human studies is Alum. Although its mechanism of effect still remains unknown, this adjuvant demonstrates an effective role in modifying Th2 or humoral responses.

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This is while this adjuvant has a weak effect in inducing cellular immune responses [7, 9]. Various studies have shown that Naloxone, an opioid receptor antagonist, has adjuvant effects [10, 14] and the ability to moderate and shift immune responses towards Th1 cellular immune responses. In addition, Naloxone can activate P38MAPK, inhibit NADPH oxidase and cause an increase in pre-inflammatory cytokines which results in inflammation [15, 18]. Cytokines have an important role in controlling viral and bacterial infections such as HIV-1; they induce their effect by moderating immune responses and directing immune response patterns in exposure to pathogens and thus defeating pathogens [19]. In the initial stages of the disease, the level of pre-inflammatory cytokines increases, but eventually the level of anti-inflammatory cytokines are increased and they become the dominant cytokines [19, 20].

Interleukin 17 is secreted from Th17 cells and its role is in causing inflammation and reinforcing the mucosal immune barrier, particularly in the gastrointestinal tract. Studies have indicated that in the progression of AIDS, an imbalance occurs among Th1, Th17/Th2, Treg immune cells which result in a reduction in the level of Th1 and Th17 and increases Th2 and Treg levels [21]. Decreased levels of IL-17 can increase the spreading of HIV virus especially in the gastrointestinal mucosa (21). Studies have indicated that a mixture of Naloxone and Alum can induce both cellular (Th1) and humoral (Th2) immune responses at a higher level [10, 14]. In addition, it has been shown that conserved and immunogenic sequences are present in the structure of HIV-1, which these protein antigens can induce protective immunity in recombinant form alone or in combination with different adjuvants and reminiscent molecules [22]. Thus it seems that co-utilization of Alum adjuvants and Naloxone with the HIV-1-gag-pol-tat-env vaccine can enhance the immunogenicity of this vaccine, and also enhance cellular and humoral immunity parameters and Th17 responses. Here we aimed to evaluate the immune

responses of BALB/c mice to HIV-1-gag-pol-tat-env recombinant protein and compare the Alum and Naloxone as adjuvants focusing on Th17 responses.

## MATERIALS AND METHODS

### *Preparation of HIV-1-gag-pol-tat-env recombinant proteins*

This protein was previously produced by Dr. Mahdavi and colleagues in the Department of Virology, Pasteur Institute of Iran and obtained for use in this study (unpublished data).

### *Vaccine formulation*

For preparation of the candidate vaccine, the recombinant protein HIV-1 Tat-pol-gag-Env was combined with Naloxone; subsequently this complex was combined with Alum adjuvant at a 1 to 4 ratio. In this combination, there was 10 µg of the candidate vaccine in every 200 µl of the prepared mixture. In addition, the amount of Naloxone in this volume was 6 milligrams/kilograms of mice. In other words, each 20 gram mouse was administered with 120 µg of Naloxone. Also, the formulation of Freund's adjuvant was prepared with the standard method and a 1 to 1 ratio of vaccine to adjuvant.

### *Experimental groups and immunization*

Experimental groups included 36 male Balb/c mice which were divided into 6 groups. Groups one to six were administered with the vaccine plus Alum and Naloxone, vaccine plus Naloxone, vaccine plus Alum, vaccine plus Freund's adjuvant, PBS, and Naloxone (negative control) respectively; Administrations were carried out on days 0, 14 and 28 subcutaneously.

### *Evaluating IgG specific titer and IgG1 and IgG2a subclasses*

For this evaluation, two weeks after the final immunization of the mice, blood samples were taken from the mice and serum was separated. In order to evaluate the titer of the serum antibodies, they were diluted 100 to 12800 times via dilution buffer and

evaluated using ELISA test. For this test, 1 µg of antigen per 100 µl of serum was kept in each well of an ELISA plate overnight at 4°C. Subsequently plates were washed and blocked with PBS containing 5% skim milk at 37 °C for one h. Then the serums (100 µl) were added for two hours at 37°C and then washed; 100 µl of mouse anti-immunoglobulin was subsequently added with a 1/10000 titer to the wells and incubated for 2h at 37°C. Finally after five washes, 100 µl of TMB substrate was added to the wells and after 30 min, by adding 100 µl of 2N Sulfuric Acid, the reaction was stopped. In addition, the level of IgG1 and IgG2a sub-classes were also determined using ELISA method. Ultimately, the results were reported as the optical density read at 450nm wavelength.

#### *Evaluating the IL17, IL-4 and IFN-γ cytokine pattern responses*

For evaluation of cytokine patterns, mice were sacrificed and their spleens were homogenized in sterile conditions in PBS. After lysis of the red blood cells via RBC lysis buffer, the cellular suspension obtained from the spleens was cultivated using RPMI+FBS 10% cultivation medium at  $3 \times 10^6$  cells/ml density in 24 well cultivation plates. Spleen cells were subsequently induced with 10 µg of specific antigen and incubated for 72h at 37°C with 5% CO<sub>2</sub>; then the supernatant was gathered and cytokines were evaluated using Mouse cytokine ELISA kit (R&D system). Results are reported as the standard curve based on picograms per milliliters (Pg/ml).

#### *Evaluating lymphocyte proliferative responses to the vaccine*

100 µl of the obtained cell suspension from spleens cultivated in RPMI+FBS 10% at  $3 \times 10^6$  cell/ml density was cultivated in a 96 well cell cultivation plate and induced with 10 µg/ml of antigen; antigen was not added to some of the wells to be used as negative control and PHA was added to some of the wells to be used as positive control. The plate was incubated for

72h at 37°C with 5% CO<sub>2</sub> and then 20 µl of Brdu was added to each well; after 18h, the plates were centrifuged for 10min at 300G. The supernatant was discarded and the plates were dried and 200µl of permeability buffer was added to the wells. After 30min of incubation, the buffer was discarded and 100µl of anti-Brdu was added to the wells and the plate was incubated at room temperature for two hours. Consequently, the plates were washed and TMB substrate was added in order to evaluate the reaction rate by analyzing the color change. The results of changes in proliferative responses are reported as induction indices with regard to the controls (absorbance of non-induced cells/absorbance of induced cells).

### STATISTICAL ANALYSIS

Initially, the mean value of the data was calculated and with regard to the data being normal, one-Way ANOVA test and Tukey test were used at 95% confidence level. All results are reported as mean ± standard deviation and P<0.05 is considered as significant.

### RESULTS

#### *Specific antibody titer*

The highest serum antibody level after three vaccinations was observed in the group administered with the vaccine plus Freund's adjuvant; groups administered with the vaccine plus Naloxone and Alum, vaccine plus Alum and vaccine plus Naloxone were next, respectively, and the control groups have lower levels. Evaluating the specific antibody level of the candidate vaccine after the second immunization shows that the antibody response levels in the group administered with the candidate vaccine plus adjuvant was significantly different from all other groups (p<0.05); in addition, injection of the vaccine plus Naloxone and Alum caused an increase in antibody response level compared to the vaccine plus Alum group, but this increase was not significant (p>0.05) (Figure1).

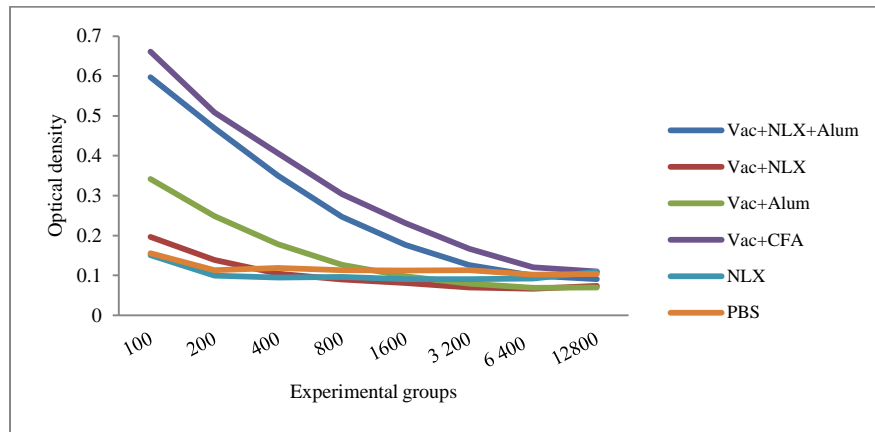


Figure 1. Evaluating serum total antibody levels in experimental groups after three immunization rounds

#### Evaluating the level of IgG1 and IgG2a antibody subclasses

The results indicated that injection of the candidate vaccine plus Alum and Naloxone adjuvants caused an increase in the level of IgG1 and IgG2 specific antibody levels which showed a significant difference with the control groups ( $P < 0.05$ ). The results of evaluating the IgG1 specific antibody level is shown that group 1 (Vaccine + Naloxone + Alum) was significantly different from group 2 (vaccine + Naloxone) ( $P = 0.027$ ), while it was not significantly different from group 3 (vaccine + Alum) and group 4 (vaccine + complete Freund's adjuvant) ( $p > 0.892$ ). Furthermore, the increase in IgG1 compared to the control groups was significant ( $P = 0.0001$ ) (Figer 2). Regarding the evaluation of IgG2a, results showed that all groups administered with the antigen were significantly different from the control groups ( $P < 0.0001$ ) (Figure 3).

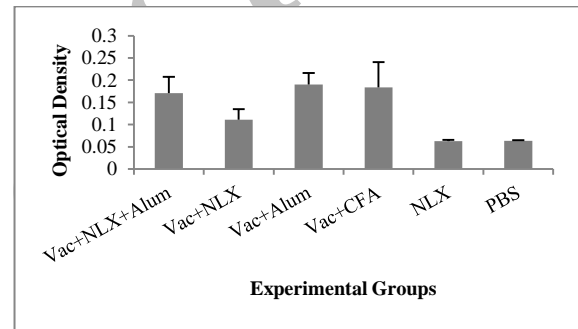


Figure 2. Evaluating IgG1 antibody level of the candidate vaccine. Data are shown as mean ± SD

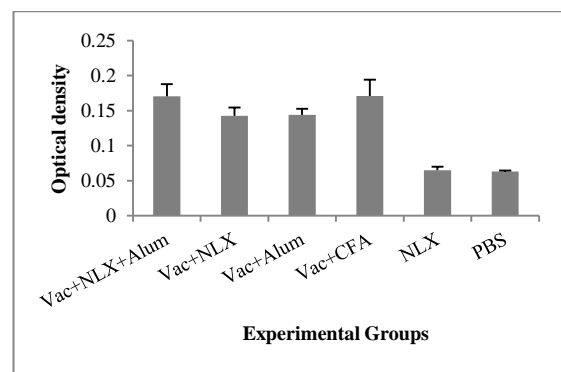


Figure 3. Evaluating IgG2a antibody level of the candidate vaccine. Data are shown as mean ± SD

### Cytokine patterns

In this study, IL-4, IL-17 and IFN- $\gamma$  cytokine levels were evaluated. Our findings demonstrated that by administering the candidate vaccine plus Alum adjuvant and Naloxone, the IL-17 level (Figure 4) is significantly increased compared to all other groups ( $P < 0.001$ ); and the results of evaluating IFN- $\gamma$  cytokine level is shown that the experimental groups had increased levels compared to the control groups ( $P < 0.041$ ). In addition, group 1 (vaccine + Naloxone + Alum) was not significantly different from group 2 ( $P = 0.132$ ), but it showed a significant increase in compare to the vaccine plus Alum and vaccine plus Naloxone groups ( $P < 0.0024$ ) (Figure 5). Regarding IL4, all groups administered with the vaccine caused a significant increase in this cytokine compared to negative control groups 5 and 6 ( $P = 0.0001$ ), although group 1 was not significantly different from any of the groups receiving the vaccine ( $P > 0.174$ ) (Figure 6).

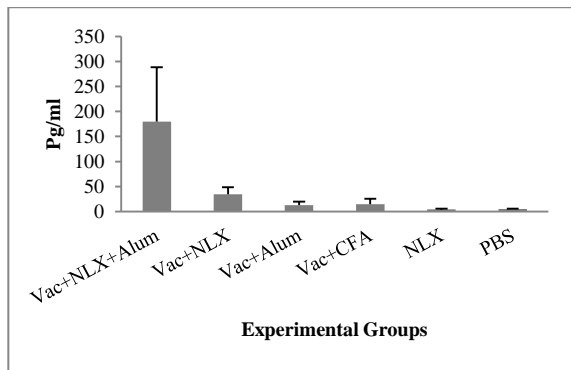


Figure 4. Evaluating IL-17 cytokine level in experimental groups. Data are shown as mean  $\pm$  SD

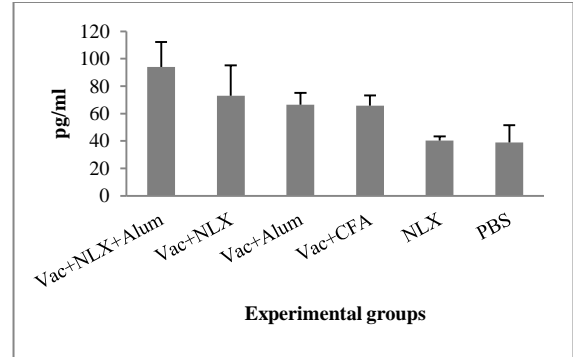


Figure 5. Evaluating IFN- $\gamma$  cytokine level in experimental groups. Data are shown as mean  $\pm$  SD

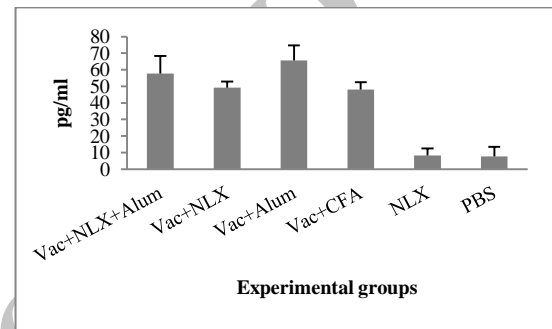


Figure 6. Evaluating IL-4 level in experimental groups. Data are shown as mean  $\pm$  SD

### Lymphocyte Proliferation levels in response to the vaccine

In this study, Brdu method was utilized to investigate spleen lymphocyte proliferative levels in response to the vaccine. The findings of this study is shown that injection of the vaccine plus Alum and Naloxone caused a significant increase in proliferation responses compared to groups 2 and 3 ( $P < 0.022$ ), although it was not significantly different from group 4 ( $P = 0.341$ ). Also, all groups administered with the vaccine were significantly different from the Naloxone and PBS groups ( $P < 0.0008$ ) (Figure 7).

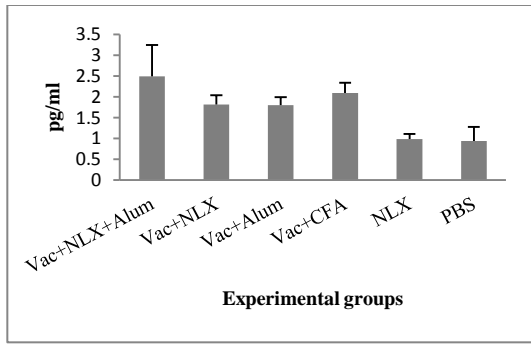


Figure 7. Result of evaluating lymphocyte proliferative responses in experimental group via Brdu method; data are shown as indices of induction and include mean  $\pm$  SD

## DISCUSSION

It is generally believed that a suitable vaccine against HIV-1 should be effective, strong and possess high quality in inducing cellular and humoral immunity (26, 28). Using adjuvants modulates and increases vaccine specific immune response levels. With regard to the use of various adjuvants in many studies, to this day, Alum has been the only adjuvant to be authorized for use in human studies [11]. Alum shifts immune responses towards Th2 and reinforces humoral immunity; the issue with the use of Alum is that this adjuvant does not sufficiently induce the cellular immune system and Th1 responses. Another adjuvant which has recently been studied and proven to be efficient is the Naloxone drug [15, 16]. In the year 2007, Jamali and et al studied the adjuvant effects of Naloxone as an opioid receptor antagonist on inducing cellular immunity in Herpes simplex 1 viral infection (HSV-1); they discovered that during the first seven days, in addition to inhibition of opioids, Naloxone causes an increase in cellular immune responses against the virus [11]. During further studies in the year 2009, it was shown that Naloxone can act as an adjuvant along with the DNA vaccine of HSV-1 to cause a shift in immune responses towards Th1 and increase the vaccine immunization against the virus; in addition, this drug prolonged the viability of the vaccinated mice [10]. During the next studies in the year 2010, Jazani and et al studied the heat-killed Salmonella

typhimurium vaccine used in combination with Naloxone as an adjuvant in Balb/c mice and evaluated it as a model for immunization against intracellular bacteria [13]. The results of this study showed that the use of Naloxone as an adjuvant increases lymphocyte proliferation, DTH responses and cellular immune responses due to a shift in immune responses towards Th1. Also, a study is conducted in the year 2011, is evaluated the adjuvant effect of the combination of Alum-Naloxone on the level of humoral and cellular immune responses in Balb/c mice administered with heat-killed Salmonella typhimurium vaccine; the results of this study indicated that the use of Alum-Naloxone combination as an adjuvant can increase the shift towards Th1, lymphocyte proliferation, induction of DTH responses, IgG, IFN- $\gamma$  and humoral and cellular immune responses which ultimately led to a decrease in mortality rate [14].

Thus it was defined that Naloxone can induce Th1 immune responses via increasing the level of IFN- $\gamma$  compared to IL-4. In addition, it was clarified that Naloxone possesses adjuvant properties which acts in increasing lymphocyte proliferative responses; plus, its combination with Alum can induce the cellular and humoral immune system, with its strongest affect in inducing the production of antibodies [10,14]. The exact mechanisms of Naloxone's function is not yet clear, but with regard to its pattern in inducing the immune system, it seems as though this drug functions at injection site by effecting local APCs, activating P38MAPK, inhibiting NOX<sub>2</sub>, production of pre-inflammatory cytokines and causing an inflammatory response [15, 18].

The results of the current study indicate that the group administered with the vaccine plus Alum and Naloxone had a significant increase in the levels of IFN- $\gamma$ , IL-4 and IL17, which show the induction of cellular immunity (Th1), Th2, and Th17 cells respectively, compared to the other groups in this study. Furthermore,

in this group, the level of IgG2a and IgG1 had a significant increase compared to other groups in this study; this increase shows the activation of Th1 and Th2 cells or in other words, activation of cellular and humoral immunity against the intended antigens. Studying lymphocyte proliferative responses in the vaccine plus Alum and Naloxone group showed a significant increase compared to the groups administered with the vaccine plus Alum, this shows this combination has had a positive effect on the proliferation of lymphocytes. The results of our study is in accordance to previous studies, indicating that the combination of Alum and Naloxone acts stronger in inducing immune responses against antigens compared to each of them administered alone; in addition, this combination can induce and increase antibody isotopes against pathogens at a higher levels, and possess an important role is defeating pathogens. Moreover, result of assessing the IL-17 level show that Naloxone, regarding its role in inflammation, has been able to affect Th17 cell and induce them to increase IL-17 levels.

#### CONCLUSION

With regard to the importance of the results obtained in this study, the significant increase in the level of immune system cells, the increase in IgG2a, IgG1, IFN- $\gamma$  and specifically IL-7 especially in the group injected with the candidate vaccine plus Alum and Naloxone compared to other groups in this study, it should be noted that Naloxone and Alum can act as an efficient adjuvant in increasing the induction of cellular immunity and specifically Th17, and shifting immune responses towards Th1 and Th17, and thus they can be applied for strengthening immune responses against virus particles in designing vaccines.

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