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# A novel adjuvant, the general opioid antagonist naloxone, elicits a robust cellular immune response for a DNA vaccine

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**Abstract**

**While many adjuvants have been discovered and used in research, only a few adjuvants have been permitted for use with human vaccination. We have previously shown that the administration of naloxone (NLX), a general opioid antagonist, during infection with a non-virulent strain of herpes simplex virus type 1 (HSV-1) could enhance protection against HSV-1 challenge. Here, the adjuvant activity of NLX has been evaluated using a DNA vaccine for HSV-1 as a model. BALB/c mice were divided into four groups; for experimental groups, mice received the glycoprotein D1 (gD1) DNA vaccine alone or in combination with the adjuvant NLX. A positive control group received the KOS strain of HSV-1, and a negative control group received PBS. All mice were immunized three times on days 0, 21 and 42. Three weeks after the last immunization, immune responses against HSV-1 were assessed. Our results indicate that the administration of NLX as an adjuvant increased the ability of the gD1 DNA vaccine to enhance cytolytic T lymphocyte activity, lymphocyte proliferation, delayed-type hypersensitivity and shifting the immune response toward a T helper ($T_h$)1 pattern and improved protective immunity against HSV-1. NLX also increased the IgG2a/IgG1 ratio, though it did not affect the production of HSV-1 antiserum. In conclusion, administration of NLX as an adjuvant in combination with the gD1 DNA vaccine can enhance cell-mediated immunity and shift the immune responses to $T_h1$.**

## Introduction

Over the last century, the use of vaccines has profoundly reduced the morbidity and mortality of infectious diseases in both human and veterinary populations. In fact, vaccination has proven to be the most successful medical intervention ever developed (1). However, currently available vaccines still fail to protect against certain viral infections, such as HIV. The prevention of these particular diseases will require the development of new vaccine technologies that can elicit a potent cellular immune response (2–4). One promising emerging technology for generating cell-mediated immunity is plasmid DNA immunization. However, while plasmid DNA immunogens can elicit cellular immune responses in small laboratory animals, these responses need to improve before DNA vaccines can be used in human clinical trials (2). Accordingly, attempts have been made to find adjuvants that enhance the immunogenicity of plasmid DNA vaccines (2, 5).

The word 'adjuvant' is derived from the Latin word 'adjuvare', which means 'to help, aid or enhance' (6). Immunological adjuvants were originally described by Ramon in 1924 as 'substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone' (7). This broad definition encompasses a very wide range of materials (8, 9).

While many adjuvants have been discovered and used in research, only a few adjuvants have been permitted for use with human vaccination. The greatest challenge with using adjuvants in human vaccinations is that most of the adjuvant formulations are associated with high toxicity and adverse side effects. At present, the adjuvant choice for human

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vaccination reflects a balance between adjuvanticity and side effects (10).

It is now clear that opioids have considerable immune-modulating effects, mediated directly by opioid receptors on immune cells and/or indirectly through the central nervous system and the hypothalamic–pituitary–adrenal axis (11). The opioid peptides act through class-specific receptors known as μ, δ and κ (11). Opioid receptors have been detected on both T and B lymphocytes, dendritic cells (DCs) and other immune cells (11). In the case of κ opioid receptors (KORs), expression is greater in lymphoid compartments (e.g. thymus) that contain immature cells. However, both δ opioid receptor (DOR) and KOR expression are greatly enhanced in activated cells. In addition, T cells show enhanced DOR expression as a function of increasing cell density, independent of activation through the TCR. Atypical binding sites for both β-endorphin and morphine have also been characterized on immune cells (12). Based on these observations, along with the anti-proliferative effects of DOR agonists and the inhibition of interferon γ (IFN-γ) production by μ opioid receptor agonists, opioid receptors appear to be part of an immunomodulatory system that responds to both opioid neuro- and immunopeptides secreted locally by innervating neurons and immune cells, respectively (12). Opioid peptides are well-known inhibitors of the immune response and vaccination against microbial agents (13–15). Furthermore, the exposure to exogenous opioids is known to increase susceptibility to microbial infection (15).

Previous works have suggested that naloxone (NLX), an opioid antagonist, can shift the immune response toward a T helper ($T_h$)1 pattern (16–18). $T_h$1 cell-mediated immunity plays a crucial role in host resistance to intracellular infection by bacteria, parasites and viruses (19). Vaccination against these pathologic agents requires a strong $T_h$1 adjuvant, such as Freund's adjuvant, which is not tolerated by humans (20). AS04 (monophosphoryle lipid A plus aluminum salts) and MF59 (an oil in water emulsion) (21), which are European Medicines Agency-approved vaccine adjuvants (22, 23), can stimulate $T_h$1 immune responses (24, 25). Aluminum salts, the only Food and Drug Administration (FDA)-approved vaccine adjuvant, enhance a $T_h$2-specific response (26). NLX is currently approved by the FDA as a prescription drug (27) and is routinely used by emergency medical personnel to rapidly and safely reverse opiate-induced respiratory depression (28). We have shown previously that the administration of NLX during primary herpes simplex virus type 1 (HSV-1) infection of a non-virulent strain could enhance the protection against HSV-1 challenge (29).

Here, we tested the ability of NLX to serve as an adjuvant for an HSV-1 DNA vaccine.

## Materials and methods

### *Mice*

Six- to eight-week old male BALB/c mice were obtained from the Pasteur institute (Karaj, Iran). Mice were housed for 1 week before the experiment, given free access to food and water and maintained in a light/dark cycle with lights on from 6:00 to 18:00 h. All experiments were in accordance with the Animal Care and Use Protocol of Urmia University of Medical Sciences.

### *Cells and viruses*

Vero cell lines were used for propagation of viruses. Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum. WEHI 164 and spleen cells were grown in Roswell Park Memorial Institute (name of the medium) (RPMI 1640) (Gibco BRL, Paisley, UK), supplemented with 10% FCS. A wild-type HSV-1 strain was isolated from a cold sore lesion of a patient. The virus was confirmed as HSV-1 with an immunofluorescence assay using an HSV-1-specific mAb (30). The neurovirulence of the virus was proven by injecting the virus into mice and then isolating the virus from brains of dead mice. HSV-1 (wild-type strain) and KOS (non-virulent strain) were grown on Vero cells, titered and stored at −70°C.

### *DNA vaccine*

Plasmid DNA encoding HSV-1 glycoprotein D1 (gD1) was constructed by insertion of the gD1 gene into pcDNA3 as described previously (30).

### *NLX treatment and immunization*

BALB/c mice were immunized three times intra-dermally with 50 μg of pcDNA3 gD1 in a total volume of 100 μl on days 0, 21 and 42 (gD1 group). NLX (Sigma) was dissolved in 100 μl PBS containing 50 μg of the pcDNA3 gD1 construct at a concentration of 5 mg $kg^{-1}$ and injected to each mouse with the same protocol, i.e. NLX was administered intra-dermally in combination with the pcDNA3 gD1 on days 0, 21 and 42 (NLX-gD1 group). PBS was injected according to the same protocol into the third group of mice as a negative control (PBS group). Finally, the last control group of mice was inoculated according to the same protocol with 100 μl of inoculums containing $10^5$ plaque-forming units (pfu) of KOS strain virus (KOS group).

### *Delayed-type hypersensitivity assay*

Virus suspensions containing $10^5$ pfu of KOS strain were UV-inactivated and injected into the right footpad of each mouse 3 weeks after the last immunization. Vero cell extract was injected into the left footpad as a negative control. The footpad thickness was measured with a dial caliper after 48 h, and the results were expressed as the mean percentage increase in the footpad thickness (in five animals). The results were calculated according to the following formula:

[(Thickness of right footpad challenged with inactive KOS) − (thickness of left footpad challenged with Vero extract)] × 100/ (thickness of left footpad challenged with Vero extract.)

### *Cytokine assays*

Three weeks after the last immunization, the spleens of individual mice were removed aseptically and homogenized in RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS and antibiotics. RBCs were osmotically lysed using ammonium chloride buffer ($NH_4Cl$ 0.16 M and Tris 0.17 M). Cells were washed twice with RPMI 1640 and counted; viability was determined by trypan blue (0.4% w/v) exclusion.

A nominal total of $1 \times 10^6$ spleen cells was plated in each well of 24-well plates using RPMI 1640 supplemented with 10% FCS, 100 IU $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin and $5 \times 10^{-5}$ M 2-mercaptoethanol. Two wells were used for each mouse. The cells were re-stimulated *in vitro* with five multiplicity of infection (MOI) of heat-inactivated virus. Plates were incubated at 37°C in 5% carbon dioxide; at 48 h after stimulation, supernatants were removed and kept at −70°C for measurement of secreted IFN-γ and interleukin 4 (IL-4) levels. The concentration of IFN-γ and IL-4 in the supernatants was estimated using a commercial ELISA Kit (R&D Systems).

*Cytotoxicity assay*

Three weeks after the last immunization, single-cell splenocyte suspensions were prepared as effector cells without *in vitro* stimulation. The WEHI 164 target cells were infected with five MOI HSV-1 (KOS) for 4 h and washed three times with assay medium. The cytolytic T lymphocyte (CTL) activity was measured with the lactate dehydrogenase (LDH) release assay in 96-well round-bottom plates (31). Target cells ($2 \times 10^4$ cells per well) in a volume of 100 μl were incubated with 100 μl of effector cells at 10/1 or 50/1 effector/target (E/T) ratios for 4 h in phenol red-free RPMI 1640 containing 3% FCS. After centrifugation, the supernatants (50 microliters per well) were transferred to 96-well flat-bottom plates, and lysis of target cells was determined by assaying LDH release with a kit (Takara Company) according to the manufacturer's instructions. Blank PBS buffer and a solution of 0.1% Triton X-100 in PBS were used as controls. The LDH-mediated conversion of the tetrazolium salt into red formazan product was measured at 490 nm after incubation at room temperature for 30 min. The percentage of specific cytolysis was determined by the following formula:

Specific cytolysis (%) = [optical density (OD) of experimental LDH release − OD of spontaneous LDH release of effector cells − OD of spontaneous LDH release from target cells]/(maximum LDH release of target cells − OD of spontaneous LDH release of target cells) × 100%. All determinations were performed in triplicate.

*Lymphocyte proliferation assay*

The lymphocyte proliferation rate was measured by using 3[4,5-dimethylthiazol-2-μl]-2,5-diphenyltetrazolium bromide; thiazolyl-blue (MTT) dye assay. Under sterile condition, spleens were removed and single-cell suspension was prepared in phenol red-free RPMI 1640 (Gibco). RBCs were lysed using 0.75% $NH_4Cl$ in Tris buffer (0.02%, pH 7.2). The concentration was adjusted to $1 \times 10^6$ cells per milliliters in phenol red-free RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 25 mM HEPES. One hundred microliters of diluted cell suspensions were dispensed into 96-well flat-bottom culture plates. Mitogen phytohemagglutinin-A at 5 μg $ml^{-1}$ final concentrations (positive control) or five MOI of heat-inactivated KOS strain was added to each well, the volume was adjusted to 0.2 ml. After incubating for 72 h at 37°C in 5% $CO_2$ humid incubator, cell proliferation was determined by MTT assay (32). Briefly, 20 μl MTT was added in each well and plates were further incubated at 37 C for 4 h. Following incubation, the supernatant from each well was aspirated carefully and formazan crystals were solubilized by adding 100 μl dimethyl sulfoxide into each well. The absorbance of each well was then determined at a wavelength of 540 nm.

*Antibody assay*

Blood samples were collected by tail bleed at 3 weeks after the last immunization. Serial 2-fold dilutions of heat-inactivated sera were prepared and mixed with equal volumes of DMEM containing 100 tissue culture infective dose 50% of KOS strain for 1 h at 37°C. The mixtures were inoculated into the Vero cells cultured in 96-well plates (Nunc, Roskilde, Denmark). The cytopathic effect was observed for 3 days. The serum titer was taken as the highest dilution of each serum capable of neutralizing HSV-1. Then ELISA assay was performed. Briefly, 96-well microtiter plates were incubated with lectin-purified HSV-1 KOS glycoprotein, used as a coating antigen (33), for 48 h at 4°C; plates were blocked with PBS containing 2% BSA (Gibco) for 2 h at 20°C. To determine the relative levels of HSV-1-specific IgG subclasses, anti-murine IgG1 and IgG2a conjugated with HRP were substituted for anti-murine IgG conjugated to HRP (Sigma). Pools composed of equal numbers of serum samples for each group were serially diluted 2-fold from 1:100 and reacted with HSV-1 KOS glycoprotein. The titers were determined as the reciprocals of the highest serum dilutions showing OD values twice as high as that of the negative control.

*Intra-peritoneal acute HSV-1 challenge*

Three weeks after the last immunization, mice were challenged with a low dose [four mouse lethal dose fifty percent ($MLD_{50}$) = $1 \times 10^5$ pfu] or a high dose (10 $MLD_{50}$ = $1 \times 10^5$ pfu) of wild-type virus. The mortality rate was followed for 2 weeks.

*Statistical analysis*

Antibody titer, delayed-type hypersensitivity (DTH) response, CTL response (LDH assay), MTT assay and cytokine production were analyzed by one-way analysis of variance followed by Tukey's test. Survival rate was measured with the Kaplan–Meier analysis and the log rank test. A $P$-value <0.05 was considered significant.

## Results

*The co-administration of NLX with gD1 DNA vaccine enhances the DTH response*

As shown in Table 1, the DNA vaccine groups (with or without NLX) and the KOS group both elicited significantly higher DTH responses than the PBS group (as expected; $P < 0.001$). Furthermore, mice immunized with either KOS or the gD1 DNA vaccine in combination with NLX exhibited a significantly higher DTH response than mice immunized with gD1 DNA vaccine alone ($P < 0.001$). There was no significant difference between the KOS group and the NLX-gD1 group.

**Table 1.** The effects of administering NLX as an adjuvant on the development of DTH

| Groups | Percentage of footpad thickness (mean ± SD) (%) |
|---|---|
| NLX-gD1 | 36.38 ± 4.89[a] |
| gD1 | 22.88 ± 3.22 |
| KOS | 39.84 ± 4.28 |
| PBS | 3.7 ± 1.10 |

BALB/c mice were immunized three times intra-dermally with pcDNA3 gD1 (gD1 group), pcDNA3 gD1 in combination with NLX (NLX-gD1 group), KOS strain (KOS group) or PBS (PBS group) on days 0, 21 and 42. Twenty-one days after final immunization, virus suspension containing $10^5$ pfu UV-inactivated KOS strain was injected into the right footpad of each mouse (five per group), while Vero cell extract was injected into the left footpad as a negative control. Values are the mean ± standard error of the mean for three experiments.
[a]Values obtained for the NLX-gD1 group were significantly different from those obtained for the gD1 group ($P < 0.001$).

*The co-administration of NLX shifts the cytokine pattern toward $T_h1$*

As shown in Fig. 1, mice immunized with the DNA vaccine or KOS produced significantly more IFN-γ and IL-4 than PBS-immunized mice ($P < 0.05$). Lymphocytes from the NLX-gD1 group and the KOS group produced the largest amounts of IFN-γ. Furthermore, the combination of NLX and gD1 DNA vaccine stimulated IFN-γ production more than gD1 DNA vaccine alone ($P < 0.001$). Lymphocytes from PBS-immunized mice secreted only very small amounts of IFN-γ and IL-4 after immunization.

While the KOS and NLX-gD1 groups had similar levels of IFN-γ, KOS-immunized mice produced significantly more IL-4 than mice immunized with the combination of NLX and gD1 DNA vaccine ($P < 0.001$). Furthermore, the NLX-gD1 group had significantly lower amounts of IL-4 than the gD1 group ($P < 0.05$).

*The co-administration of NLX with gD1 DNA vaccine enhances the CTL response*

The CTL response in immunized mice was examined using the LDH release assay. As shown in Fig. 2, the CTL response was significantly higher in mice treated with the DNA vaccine and KOS than in those treated with PBS ($P < 0.05$). Furthermore, the KOS and NLX-gD1 groups had a significantly higher CTL response than the group treated with gD1 DNA without NLX ($P < 0.001$). There was no significant difference in the CTL responses of the KOS group and NLX-gD1 groups.

*The co-administration of NLX with gD1 DNA vaccine enhances lymphocyte proliferation*

Since lymphocyte proliferative responses are generally considered as a measure of cell-mediated immunity, HSV-1 antigen-specific lymphocyte proliferation was evaluated using MTT assay. As shown in Fig. 3, the lymphocyte proliferation was significantly higher in mice treated with the DNA vaccine and KOS than in those treated with PBS ($P < 0.05$). Furthermore, the KOS and NLX-gD1 groups had a significantly higher lymphocyte proliferation than the group treated with gD1 DNA without NLX ($P < 0.001$). There was no significant difference in the lymphocyte proliferation of the KOS group and NLX-gD1 groups.

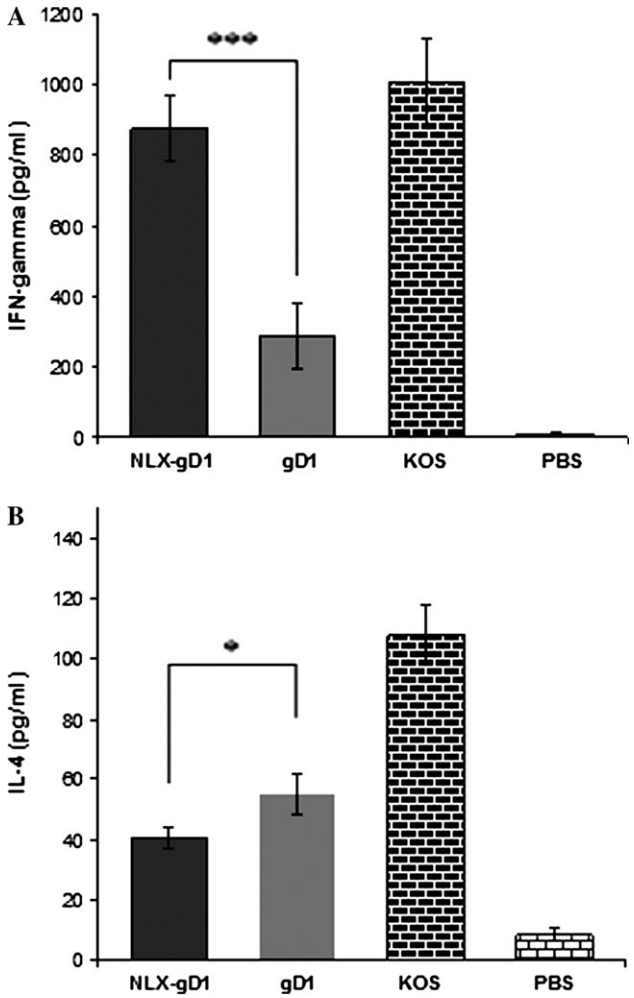


**Fig. 1.** The effect of administering NLX on IFN-γ and IL-4 production. BALB/c mice were immunized three times intra-dermally with pcDNA3 gD1 (gD1 group), pcDNA3 gD1 in combination with NLX (NLX-gD1 group), KOS strain (KOS group) or PBS (PBS group) on days 0, 21 and 42. Twenty-one days after final immunization, spleens of individual mice (five per group) were removed and IFN-γ and IL-4 production was measured with an ELISA Kit. (A) Mice of the NLX-gD1 group had significantly more IFN-γ than those of the gD1 group (***$P < 0.001$). (B) Production of IL-4 in mice of the NLX-gD1 group was significantly lower than in mice of the gD1 group (*$P < 0.05$). Values are the mean ± standard error of the mean for five experiments.

*HSV-1 antibody response*

Neutralizing antibody titer and IgG production were measured at 21 days after the third immunization, and the results are shown in Table 2. Both the DNA vaccine groups and the KOS group exhibited higher antibody responses and IgG production than the PBS group ($P < 0.001$). KOS-immunized mice had higher levels than mice immunized with DNA vaccine with or without NLX ($P < 0.001$). No significant difference was observed between the gD1 and NLX-gD1 groups. Also, the subclasses of IgG that were

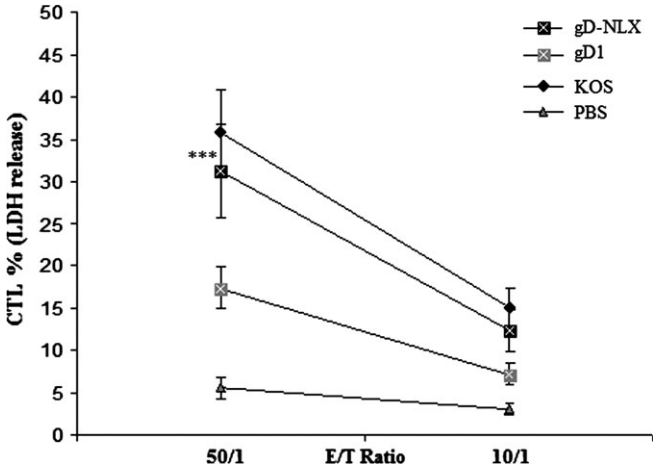


**Fig. 2.** CTL activity in immunized mice. BALB/c mice were immunized three times intra-dermally with pcDNA3 gD1 (gD1 group), pcDNA3 gD1 in combination with NLX (NLX-gD1 group), KOS strain (KOS group) or PBS (PBS group) on days 0, 21 and 42. Twenty-one days after final immunization, splenocytes from immunized and mock-immunized mice were prepared as described in Materials and Methods. LDH release assays were performed in triplicate with splenocytes as effector cells and HSV-1 (KOS strain) WEHI 164 cells as target cells. All experiments were performed more than three times, and each group consisted of five mice. The E:T cell ratio was 50:1. CTL activity of the NLX-gD1 group was significantly higher than that of the gD1 group (***$P < 0.001$). E:T ratio, effector-to-target cell ratio.

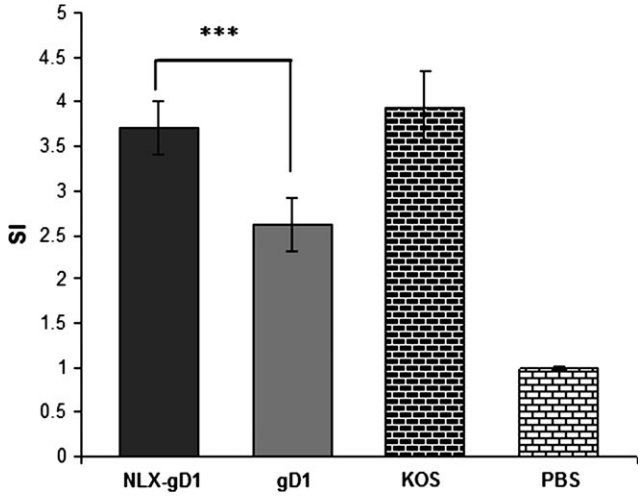


**Fig. 3.** The effect of administering NLX on lymphocyte proliferation. BALB/c mice were immunized three times intra-dermally with pcDNA3 gD1 (gD1 group), pcDNA3 gD1 in combination with NLX (NLX-gD1 group), KOS strain (KOS group) or PBS (PBS group) on days 0, 21 and 42. Twenty-one days after final immunization, spleens of individual mice (five per group) were removed and lymphocyte proliferation was evaluated with MTT method. Values are the mean ± standard error of the mean for five experiments. Lymphocyte proliferation of the NLX-gD1 group was significantly higher than that of the gD1 group (***$P < 0.001$).

induced with the co-injections were analyzed (Fig. 4A). As shown in Fig. 4(B), the IgG2a/IgG1 ratios of the NLX-gD1 group were significantly higher than the gD1 group ($P < 0.01$).

**Table 2.** Antibody response and IgG production in studied groups

| Groups | No. of responder | Neutralization titer (log10) | IgG titer(OD) |
|---|---|---|---|
| NLX-gD1 | 5/5[a] | 2.15 ± 0.11[b] | 0.59 ± 0.04[b] |
| gD1 | 5/5 | 2.26 ± 0.03 | 0.61 ± 0.02 |
| KOS | 5/5 | 2.89 ± 0.07[c] | 1.86 ± 0.23[c] |
| PBS | 0/5 | ⩽0.75 | 0.09 ± 0.05 |

BALB/c mice were immunized three times intra-dermally with pcDNA3 gD1 (gD1 group), pcDNA3 gD1 in combination with NLX (NLX-gD1 group), KOS strain (KOS group) or PBS (PBS group) on days 0, 21 and 42. Blood samples were collected at 21 days after final immunization by tail bleeding. The means of serum-neutralizing antibody titers (five animals) or IgG production were measured using collected individual serum. Values are the mean ± standard error of the mean for three experiments.
[a]Minimal titer of antibody response is equivalent to the half dilution of serum that neutralized 100 tissue culture infective dose 50% of HSV-1.
[b]Mice of the KOS group showed a significantly higher level of antibody response and IgG production than those of the gD1 and NLX-gD1 groups.
[c]There was no significant difference between the antibody response and IgG production of the gD1 and NLX-gD1 groups.

### *The co-administration of NLX with the gD1 DNA vaccine partially enhances protective immunity against HSV-1 challenge*

At a low-dose rate of HSV-1 challenge, mice of both the KOS and NLX-gD1 groups were completely protected against HSV-1 infection while those of gD1 group were only 80% protected (Fig. 5A). At a high-dose rate of challenge, KOS-immunized mice had a significantly lower mortality rate in comparison to gD1 DNA vaccine-immunized mice ($P < 0.05$). This rate was lower in the KOS group than in the NLX-gD1 group, but the difference was not significant (Table 3, Fig. 5B).

## Discussion

We investigated the ability of NLX to enhance the protection of a DNA vaccine against HSV-1 infection. DNA vaccination is an attractive immunization technique for the induction of cellular immune responses required for the prevention of various infectious diseases, such as HIV, tuberculosis, leishmaniasis and HSV. However, these responses need further improvement before they reach human clinical trials.

The KOS strain of HSV-1 can be used as a live vaccine in mice that strongly induces both humoral and cellular immunity against acute HSV-1 infection (33). However, because a live HSV virus can undergo latency (34), this model of immunization is not suitable for use in humans. Since a live HSV vaccine is likely to induce a good immune response against HSV-1 infection, however, we felt it was a good model for comparing the ability of NLX to serve as an adjuvant in eliciting an immune response when administered with a DNA vaccine.

Our results show that mice of all three experimental groups—NLX-gD1, gD1 and KOS—had significantly higher CTL activity, DTH reactions, lymphocyte proliferation and production of IFN-γ, IL-4 and antibody than mice of the PBS group. However, CTL activity, DTH reaction, lymphocyte proliferation and IFN-γ production were significantly higher in

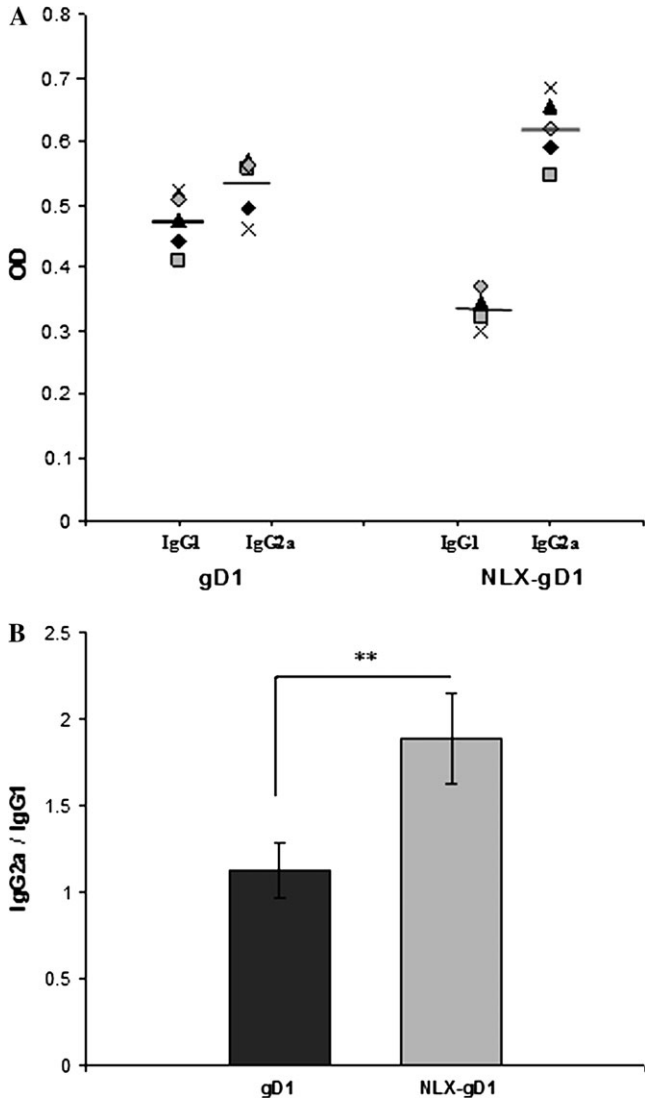


**Fig. 4.** IgG2a versus IgG1 subclass ratios in mice immunized with NLX-gD1 DNA vaccine or gD1 DNA vaccine. BALB/c mice were immunized three times intra-dermally with pcDNA3 gD1 (gD1 group), pcDNA3 gD1 in combination with NLX (NLX-gD1 group), KOS strain (KOS group) or PBS (PBS group) on days 0, 21 and 42. The mice ($n$ = 5 per group) were bled 3 weeks after the last immunization, and sera in each group were equally pooled and diluted to 1:100 for a reaction with HSV-1 glycoproteins. HSV-1-specific IgG1 and IgG2a were measured using ELISA. Horizontal lines in each group indicate the mean of their ODs (A). Values and error bars represent the means and standard deviations, respectively. The IgG2a/IgG1 ratio was calculated by dividing the mean OD of IgG2a by that of IgG1 (B). Values are the mean ± standard error of the mean for three experiments. IgG2a/IgG1 ratios of the NLX-gD1 group were significantly higher than those of the gD1 group (**$P$ < 0.01).

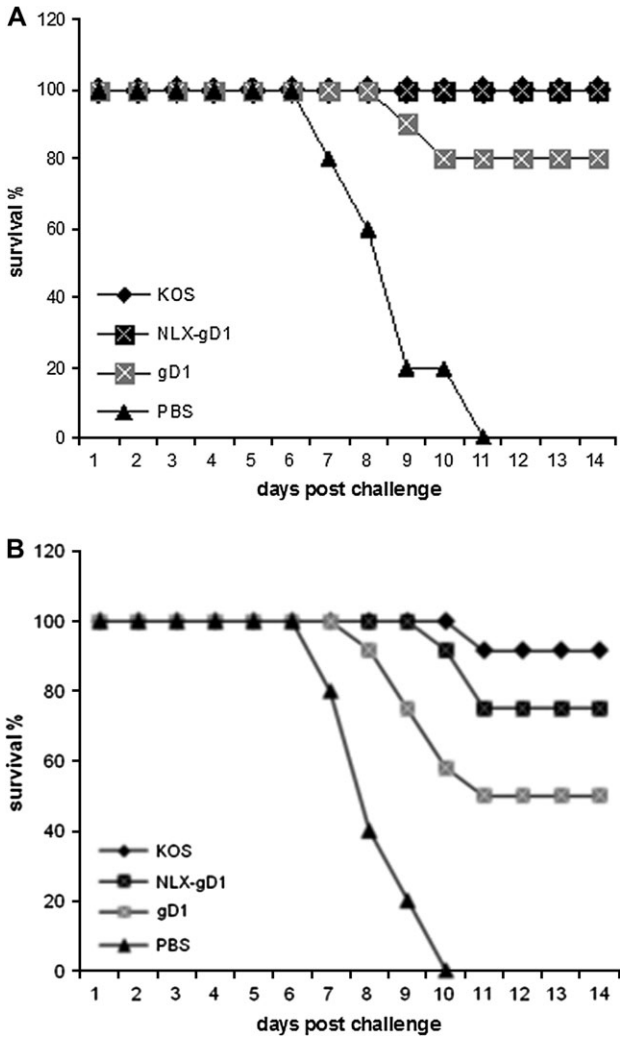


**Fig. 5.** Survival of NLX-gD1, gD1, KOS and PBS-immunized mice after acute HSV-1challenge. BALB/c mice were immunized three times intra-dermally with pcDNA3 gD1 (gD1 group), pcDNA3 gD1 in combination with NLX (NLX-gD1 group), KOS strain (KOS group) or PBS (PBS group) on days 0, 21 and 42. Twenty-one days after final immunization, mice were infected with low dose (A) or high dose (B) of acute HSV-1 strain. Survival rate was recorded daily for 14 days.

the NLX-gD1 and KOS groups than in the gD1 group. Furthermore, administration of NLX as an adjuvant in combination with gD1 DNA vaccine induced significantly lower levels of IL-4 production than the gD1 DNA vaccine alone. IgG2a/IgG1 ratios were significantly higher in the NLX-gD1 group than in the gD1 group. These results suggest that the administration of NLX in combination with a gD1 DNA vaccine increases the ability of the gD1 DNA vaccine to induce cellular immunity and shifts the immune response toward a $T_h1$ pattern.

The shift to a $T_h1$ pattern plays an important role in the induction of cellular immunity (19). Previous work has shown that the concentration of β-endorphin, an endogenous opioid in PBMC, increases under conditions in which the $T_h2$ cytokine pattern prevails, for example in cells obtained from HIV-positive subjects (35). Sacerdote *et al.* have shown that both acute and chronic NLX administration shifts the immune system toward a $T_h1$ response (18). Furthermore, we showed previously that administration of NLX during primary HSV-1 infection with a non-virulent strain could significantly increase the cellular immunity and production of IFN-γ against HSV (29). The difference here is the duration of exposure to NLX. Previous studies (18, 29) used a longer exposure to NLX and a longer period of opioid receptor

**Table 3.** Survival rates after challenge with wild HSV-1 strain

| Groups | No. of mice survived/no. of mice challenged | Average time of death ± SD (days) | Percentage of survival (%) |
|---|---|---|---|
| NLX-gD1 | 9/12 | 13.17 ± 0.42 | 75.0 |
| gD1 | 6/12 | 11.75 ± 0.67 | 50 |
| KOS | 11/12 | 13.75 ± 0.23[a,b] | 91.7 |
| PBS | 0/5 | 8.4 ± 0.51 | 0.00 |

BALB/c mice were immunized three times intra-dermally with pcDNA3 gD1 (gD1 group), pcDNA3 gD1 in combination with NLX (NLX-gD1 group), KOS strain (KOS group) or PBS (PBS group) on days 0, 21 and 42. Twenty-one days after final immunization, mice were infected with a high dose (10 $MLD_{50}$) of wild HSV-1 strain. Survival rate was recorded daily for 14 days. These results are summaries of two to three experiments per treatment regimen. These data show the mean survival rates of each group after challenge with a high dose of wild HSV-1.

[a]KOS-immunized mice showed a significantly lower mortality rate compared with gD1 DNA vaccine-immunized mice ($P < 0.05$).

[b]The mortality rate of the KOS group was lower than that of NLX-gD1 group, but this difference was not significant.

blocking in comparison to current study, which used only one time administration of NLX associated with every immunization. Therefore, the stimulatory effect of NLX on cellular immune response and the induction of the $T_h1$ shift seen in these previous studies could be direct effects of NLX on innate and specific immune responses, i.e. via binding to cells of innate and specific immune systems, respectively.

Previous studies have been shown that after injection of DNA vaccine, antigen-presenting cells (APCs) attracted to the site of injection may take up the DNA, express the encoded protein and present it to T cell (36–38). Furthermore, these studies showed some adjuvants such as CpG motifs stimulated induction of innate immunity by these APCs during uptake of DNA vaccine and shift adaptive immunity toward $T_h1$. In the current study, however, considering the half-life of NLX (39), the results observed could only be effects of NLX on the innate immune response, which in turn influence the specific immune response; ultimately, the specific immune response is triggered by NLX indirectly, i.e. without binding of NLX to its receptors on T and/or B lymphocytes. Inhibition of inflammatory responses by endogenous opioids and its acceleration by NLX has been previously showed (40–43). Therefore, one possible mechanism of NLX action is to provide a pro-inflammatory milieu by blocking opioid receptors. This inhibition would accelerate local inflammation via a direct effect on cells of innate immunity (e.g. monocytes/macrophages). This proposed mechanism is supported by the finding that incubation of DCs with κ-specific agonists causes a specific and concentration-dependent decrease in their ability to stimulate allogeneic T cells (44). Moreover, it was shown that the *in vitro* addition of the specific μ opioid receptor agonist DAMGO and the κ-specific agonist U-50488 to a culture of murine peritoneal macrophages reduced IL-12 without affecting IL-10. These effects were reversed by administration of naltrexone, an opioid receptor antagonist (45). NLX may trigger inflammation by increasing the release of local pro-inflammatory neuropeptides, for example substance P (SP) from nerve fibers (46–48). In such a pro-inflammatory milieu, APCs could polarize specific immune responses toward cellular immunity and a $T_h1$ pattern. Furthermore, it has been shown that pro-inflammatory neuropeptides (e.g. SP) stimulate maturation and migration of local APCs to draining lymph nodes and shift immune responses toward a $T_h1$ pattern (46, 49).

Hence, administration of NLX in combination with a gD1 DNA vaccine, may activate the APCs via above-mentioned mechanisms; therefore, the proteins encoded by the DNA vaccine will be presented by activated APCs. Therefore, however, the half-life of NLX is too short and probably while the proteins encoded by the DNA vaccine are presented, there is not any NLX in the environment, but previously activated APCs could induce a $T_h1$ immune response.

In the case of the gD1 antigen of HSV-1, the induction of a $T_h1$ immune response due to co-administration of NLX resulted in greater CTL activity, lymphocyte proliferation, a DTH reaction and IFN-γ production when the primed T lymphocytes were exposed again to HSV-1 (50).

The KOS group had the highest antiserum against HSV-1 of all groups. This result is consistent with previous studies showing that KOS immunization induced a significantly higher humoral immune response than DNA vaccine immunization (51).

This result may be explained by the fact that the live vaccine strongly stimulates both arms of the immune system, while the NLX adjuvant only shifts the immune responses to $T_h1$ to improve the cellular immune response.

The $T_h1$ versus $T_h2$ nature of the induced immune responses can be assessed by examining the subclasses of IgG. It is known that IgG1 is a $T_h2$-associated antibody, whereas IgG2a is a $T_h1$-associated antibody (52). Here, we observed that the IgG2a/IgG1 ratios were significantly higher in the NLX-gD1 group than in the gD1 group. This finding is in agreement with our previous data showing that the administration of NLX with the gD1 DNA vaccine increased the ability of gD1 DNA vaccine in shifting the immune response to $T_h1$.

Mice treated with NLX plus the gD1 DNA vaccine had a higher rate of survival upon challenge with wild HSV-1 than those mice given the gD1 DNA vaccine without NLX. Mice vaccinated with the KOS strain, however, had an even higher rate of survival, a result that may be explained by, as mentioned above, the ability of the KOS strain vaccine to stimulate humoral immunity. $CD4^+$ T cells and IFN-γ have important roles in the induction of the CTL response and the resulting lysis of infected cells and restriction of viral replication. IFN-γ can overcome HLA class I down-regulation and the enhancement of the CTL response against HSV-infected cells (34). Hence, increasing IFN-γ production through NLX treatment can lead to an increase in viral clearance and protection against acute HSV-1 infection. While cell-mediated immunity is the major protective immune defense against HSV-1, humoral immunity has an important role in the restriction of HSV-1 replication and spread to the central nervous system (53). Hence, the NLX-gD1 group showed only a partial enhancement of protection against a high dose of HSV-1 challenge, compared with the KOS group.

As above mentioned, it has been shown that acute exposure to exogenous opioids results in suppression of various

immune responses. On the other hands, some immune parameters, which are suppressed during acute exposure to exogenous opioids, return to normal levels as the time of exposure to the drug is increased, i.e. tolerance to the effects of opioids can develop in the immune system (54). It has been shown that withdrawal following chronic administration of exogenous opioids leads to immunosuppression (54, 55). Activation of some neural pathways, including sympathetic nervous system, which modify the immune system, is among suggested mechanisms for immunosuppression following exogenous opioid withdrawal (54, 56). The exogenous opioid withdrawal may be precipitated by an opioid receptor antagonist such as NLX or naltrexone. It seems that in such a condition, the immunosuppression due to opioid withdrawal will overcome any possible immunostimulatory effects of NLX or naltrexone. Furthermore, it has been shown that in the opposite of short-term administration of opioid antagonists, chronic administration of opioid antagonists, which is usually used in opioid withdrawal, not only does not stimulate the inflammatory responses but also may even suppress the inflammation (57). Our experiments (A. Jamali, M. Mahdavi, Z. M. Hassan, F. Sabahi, M. J. Farsani, M. Parsania, T. Bamdad, and S. Shahabi, unpublished data) confirm this finding too.

In conclusion, administration of NLX as an adjuvant in combination with the gD1 DNA vaccine can enhance cell-mediated immunity and shift the immune responses to $T_h1$. As NLX is an approved drug for human use (27), it may be a new, relatively safe, adjuvant to elicit effective vaccine-induced immune responses to malignancies and microbes that are combated with $T_h1$ immune responses. There are other adjuvants evaluated to increase ability of DNA vaccines to stimulate immune system, for example delivery of DNA vaccine in association with liposomes and similar lipid formulations. Vaxfectin (Vical Inc., a cationic and neutral lipid formulation) has proven effective in enhancing humoral and cellular immune responses to plasmid DNA immunization but does not change the $T_h1/T_h2$ profile (58–60). Therefore, it seems that for shifting immune responses toward $T_h1$ pattern, NLX is a more suitable adjuvant than Vaxfectin. Formulation ability is another advantage of NLX. On the other hand, Vaxfectin potentially increases antibody production and adjuvanting low DNA vaccine doses (58, 59). Hence, combined use of NLX and Vaxfectin may be useful to be investigated in future studies. Up to our knowledge, this study is the first of its kind to be carried out; therefore, follow-up studies are needed to confirm these results and test the adjuvant activity of NLX when combined with vaccines against other microbes that are fought with $T_h1$ immune responses.

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## Abbreviations

| | |
|---|---|
| APC | antigen-presenting cell |
| CTL | cytolytic T lymphocyte |
| DC | dendritic cell |
| DOR | δ opioid receptor |
| DTH | delayed-type hypersensitivity |
| FDA | Food and Drug Administration |
| gD1 | glycoprotein D1 |
| HSV-1 | herpes simplex virus type 1 |
| IFN-γ | interferon γ |
| IL-4 | interleukin 4 |
| KOR | κ opioid receptor |
| LDH | lactate dehydrogenase |
| $MLD_{50}$ | mouse lethal dose fifty percent |
| MOI | multiplicity of infection |
| MTT | 3[4,5-dimethylthiazol-2-μl]-2,5-diphenyltetrazolium bromide; thiazolyl-blue |
| NLX | naloxone |
| OD | optical density |
| pfu | plaque-forming units |
| RPMI 1640 | Roswell Park Memorial Institute (name of the medium) |
| SP | substance P |
| $T_h$ | T helper |

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