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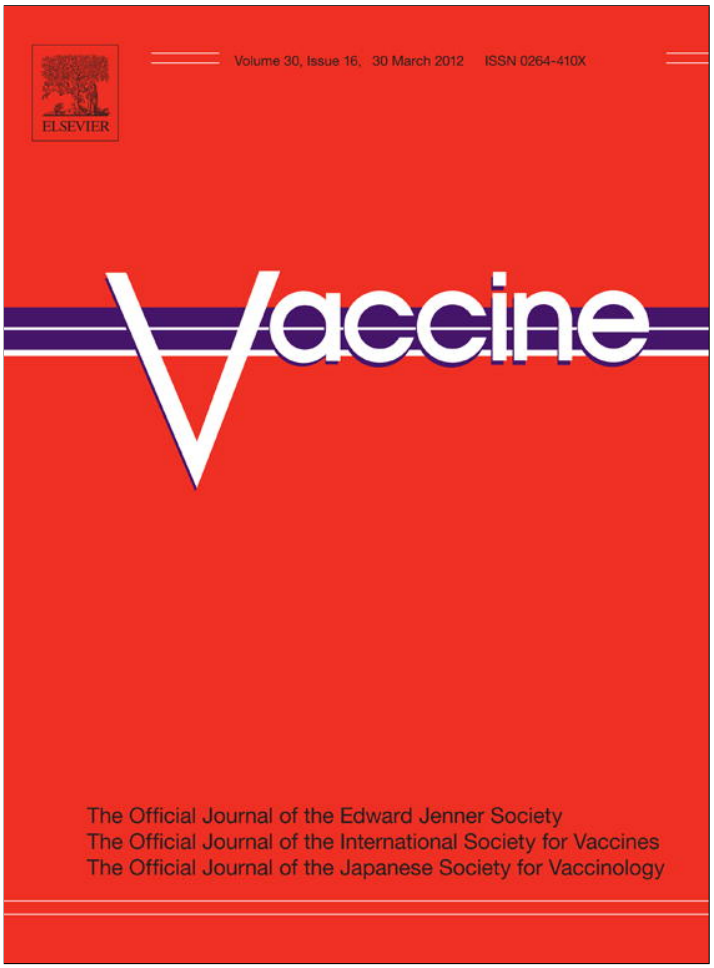

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A novel adjuvant, mixture of alum and the beta-adrenergic receptor antagonist propranolol, elicits both humoral and cellular immune responses for heat-killed *Salmonella typhimurium* vaccine

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ABSTRACT

Objective: To determine the efficacy of the mixture of propranolol (PRP), a beta-adrenergic receptor antagonist, and alum, as a new adjuvant, in the induction of humoral and cellular immunity in response to heat-killed *Salmonella typhimurium (S. typhimurium)* (HKST) as a model vaccine.
Methods: BALB/c mice were divided into five groups. Mice in the experimental groups received either the HKST vaccine alone or in combination with the adjuvant alum, PRP or the alum–PRP mixture. Mice in the negative control group received phosphate-buffered saline. All mice were immunized two times on days 0 and 14. Two weeks after the last immunization, immune responses to *S. typhimurium* were assessed.
Results: Administration of the alum–PRP mixture as an adjuvant increased the ability of the HKST vaccine to enhance lymphocyte proliferation, shifted the immune response towards a T-helper (Th) 1 pattern and increased *S. typhimurium* specific IgG, IgG2a and IgG1. This resulted in improved protective immunity against *S. typhimurium*.
Conclusion: Administration of the alum–PRP mixture as an adjuvant in combination with the HKST vaccine, can enhance both humoral and cellular immunity and shift the immune responses to a Th1 pattern.

1. Introduction

Although vaccination has profoundly reduced the morbidity and mortality caused by infectious diseases in both human and animal populations [1], vaccines that are currently available still fail to protect against certain pathogens.

One promising strategy for addressing this challenge is the development of new vaccine adjuvants that enhance the effectiveness of vaccines [2]. These adjuvants should have the ability to elicit a potent immune response. The most effective immune response against multiple pathogens involves a combination of both humoral and cellular components. This is even true for some obligate intracellular pathogens [3,4]. Safety is another important parameter to consider when choosing an appropriate adjuvant, especially for human vaccination [5]. A large number of adjuvants have been used in animal models, however the vast majority of these are not suitable for use in humans because of their toxicity [6]. The only vaccine adjuvant that is approved by the United States Food and Drug Administration (FDA) is alum (aluminum-based mineral salt) [6]. In addition to alum, the oil-in-water emulsions MF59 and AS03 and the monophosphoryl Lipid A formulated in alum (AS04) have been approved by the European Medicines Agency as vaccine adjuvants [6–8]. However, alum remains the only adjuvant approved worldwide for human use [9]. Alum has safely been used widely and successfully in many licensed vaccines. It is considered the adjuvant of choice for vaccines against infectious diseases that can be prevented by the humoral immune response [9,10]. However, some limitations of alum have been described. The major limitations of alum is its inability to elicit cell-mediated T helper1 (Th1) responses that are required to control most intracellular pathogens [9,11].

Evidence indicates that sympathetic nervous system modulates immune reactions not only by circulating catecholamines secreted by the adrenal medulla but also through innervation of all lymphoid organs and immune cells. Beta-adrenergic receptor (beta-ARs) agonists, especially beta2-ARs agonists, promote immune responses

Abbreviations: PRP, propranolol; *S. typhimurium*, *Salmonella typhimurium*; HKST, heat-killed *Salmonella typhimurium*.

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toward T helper 2 (Th2) profile. This effect is due to stimulation of beta-ARs on antigen presenting cells (APCs) and T lymphocytes [12,13].

We previously showed that it is possible to robust vaccine-induced immune responses against viruses and bacteria via a neuroimmunological intervention using naloxone, an opioid receptor antagonist, or alum–naloxone mixture as vaccine adjuvants [14–18]. In the current study, we tested the hypothesis that propranolol (PRP), a pan beta-blocker, alone or in mixture with alum can be used as an adjuvant for heat-killed *Salmonella typhimurium* (*S. typhimurium*) (HKST). We used HKST as a vaccine model against facultative intracellular bacteria that require both humoral and cellular immunity for proper host protection [19]. Since immunogenic doses of HKST without adjuvant may efficiently stimulate immune responses [20], we used a sub-immunogenic dose of HKST to evaluate the adjuvant activity of the mixture of alum and PRP.

2. Material and methods

2.1. Mice

Six- to eight-week-old male BALB/c mice were obtained from the Razi Institute (Karaj, Iran). One week prior to the experiments, the mice were allowed to acclimate to housing conditions. All experiments were conducted in accordance with the Animal Care and Use Protocol of Urmia University of Medical Sciences (Urmia, Iran).

2.2. Preparation of HKST

S. typhimurium Persian Type Culture Collection 1735 was grown on blood agar plates (Merck, Germany) overnight at 37 °C. Cultures were then harvested, centrifuged and washed three times in phosphate-buffered saline (PBS). The recovered bacteria were resuspended in phosphate buffer saline (PBS) and incubated at 80 °C for 2 h to generate a heat-killed preparation. An absence of viable colonies was confirmed by the lack of bacterial growth on blood agar plates. The bacterial concentration was enumerated by comparing the absorbance of a serial dilution of HKST at 590 nm with McFarland Nephelometer Standards. The HKST was then stored at −70 °C. The optimal dose for immunization was determined by preliminary titration (data not shown).

2.3. Immunization protocol

The alum–PRP mixture was prepared by thoroughly mixing 50 μL of PBS containing PRP (Sigma, Germany) at a concentration of 3 mg/kg with 50 μL of alum (aluminum phosphate gel, Sigma). The mixture was incubated for 72 h at 4 °C under sterile conditions. HKST (10^6) suspended in 50 μL PBS absorbed in 100 μL of the alum–PRP mixture was injected subcutaneously into the mice in the Al-PRP-Vac group. The HKST was also suspended in 100 μL of PBS that was absorbed in 50 μL of alum. This preparation was injected subcutaneously into the mice in the Al-Vac group. The mice of the PRP-Vac group received a subcutaneous injection of the HKST suspended in 100 μL of PBS plus PRP (3 mg/kg), and then dissolved in 50 μL of PBS. The mice of Vac group received 50 μL of HKST suspended in 100 μL of PBS subcutaneously. The mice that were part of the control group received a subcutaneous injection of 150 μL of PBS. So all of the mice were injected with a total volume of 150 μL. Immunization was performed twice: days 0 and 14.

2.4. Cytokine assays

Two weeks after the last immunization, the spleens of five mice per group were removed aseptically and homogenized in RPMI 1640 medium (Sigma, Germany) supplemented with 10% fetal cow serum (FCS) (Gibco-BRL) and antibiotics. Red blood cells (RBCs) were then osmotically lysed using ammonium chloride buffer (NH_4Cl 0.16 M, Tris 0.17 M). The cells were washed twice with RPMI 1640 and counted, with viability determined by trypan blue (0.4%, w/v) exclusion. A nominal total of 1×10^5 spleen cells were plated in each well of a 24-well plate using RPMI 1640 that was supplemented with 10% FCS, 100 IU mL^{-1} penicillin, 100 mg mL^{-1} streptomycin and 5×10^{-5} M 2-mercaptoethanol. Two wells were used per mouse. The cells were restimulated in vitro with 4.5×10^4 HKST. The optimal dose of HKST for restimulation had been determined by preliminary titration. Plates were then incubated at 37 °C in 5% carbon dioxide (CO_2). The supernatants were removed 72 h after stimulation and stored at −70 °C. The concentration of secreted interferon-γ (IFN-γ) and interleukin-5 (IL-5) levels in the supernatants was estimated using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Bender Med Systems, Vienna).

2.5. Lymphocyte proliferation assay

Two weeks after the last immunization, the lymphocyte proliferation rate was measured using an MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl-blue, Sigma] dye assay. The spleens of five mice per group were removed under sterile conditions and single-cell suspensions were prepared in RPMI 1640 medium (Sigma, Germany). RBCs were lysed using 0.75% ammonium chloride in Tris buffer (0.02%, pH 7.2). The concentration was adjusted to 1×10^6 cells mL^{-1} in RPMI 1640 that was 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 (Gibco-BRL) at a final concentration of 5 mg mL^{-1} (positive control) or 4.5×10^4 HKST was added to each well and the volume was adjusted to 0.2 mL. After incubating for 48 h at 37 °C in 5% CO_2, cell proliferation was determined using an MTT assay [21]. Briefly, 20 μL of MTT was added to each well and the plates were further incubated at 37 °C for 4 h. Following incubation, the supernatant was carefully aspirated from each well and formazan crystals were solubilized by adding 100 mL of dimethyl sulfoxide (DMSO). The absorbance of each well was then determined at a wavelength of 540 nm. Stimulation indices were determined and expressed as differences between the absorbance of treated and untreated wells.

2.6. Determination of total IgG titers and IgG isotyping

Two weeks after the last immunization, the levels of IgG antibodies were measured in the sera of five mice from each group by ELISA using 96-well microtiter plates [22]. The optimum dilution of the sera and the optimum dose of HKST to be used in the ELISA were determined using the checkerboard assay. Then, 200 μL of antigen (HKST 5×10^6 per 200 μL) in the coating buffer (0.1 M carbonate, pH 9.5) was added to each well of a 96-well microtiter plate. Coated plates were incubated at 4 °C overnight, washed with PBST (PBS with 0.05% Tween 20) three times and blocked with 5% bovine serum albumin in PBST for 2 h at 37 °C. After washing the plates with PBST, different dilutions (200 μL) of sera were added to the wells. Plates were incubated at 37 °C for 2 h. After washing three times with PBST, the plates were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma), IgG1 or IgG2a (Serotec). After washing with PBST three times, the reaction was developed by adding 200 μL of a TMB/H_2O_2 substrate. The reaction was terminated by the addition of 50 μL of $2NH_2SO_4$ and the absorbance was read at 450 nm wavelength.

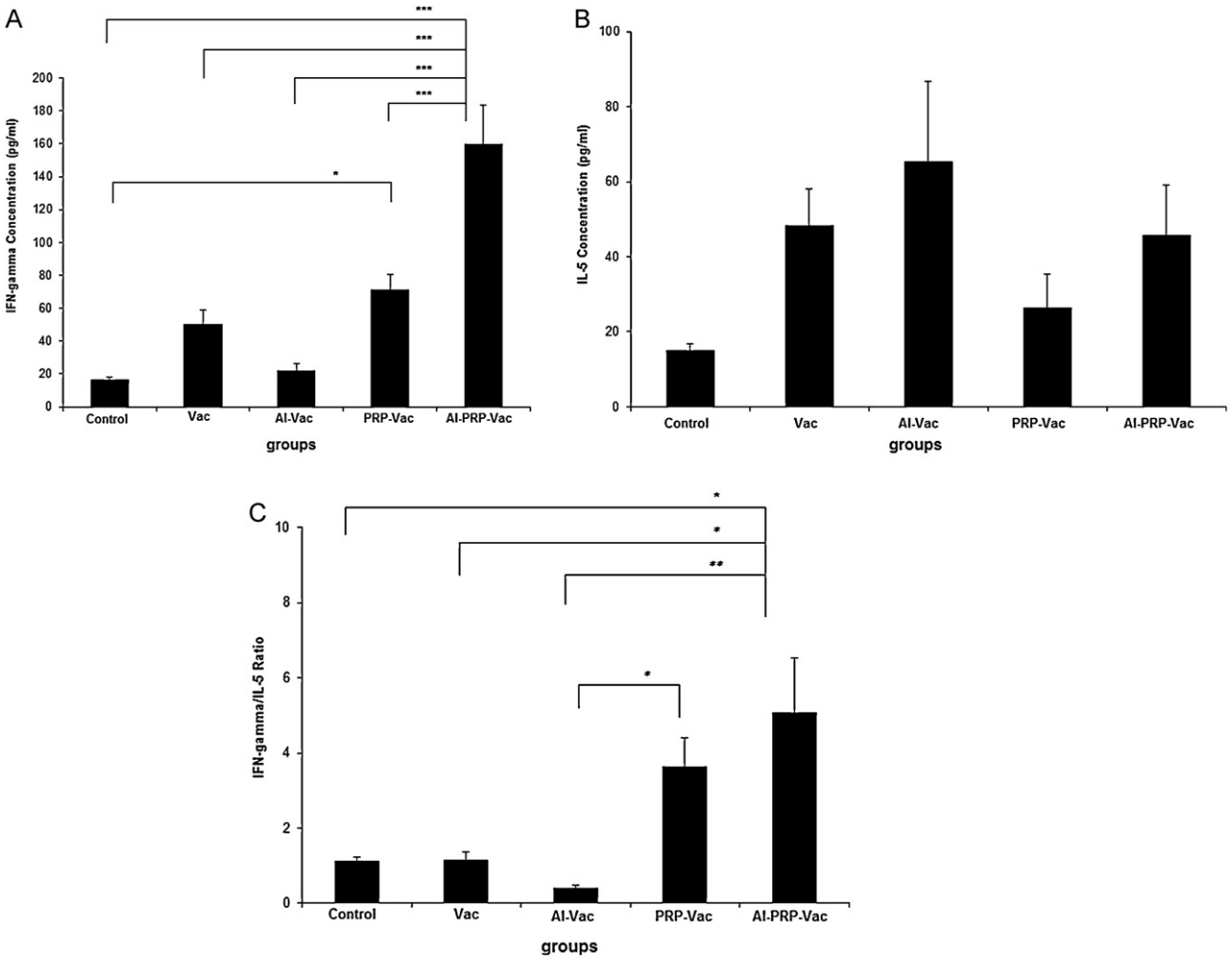

Fig. 1. Effect of administering alum–PRP mixture on IFN-γ and IL-5 production. Two weeks after the final immunization, the spleens of individual mice (five per group) were removed and IFN-γ (A) and IL-5 (B) production and IFN-γ/IL-5 ratio (C) were measured and compared between Al-PRP-Vac (alum–PRP mixture in combination with the HKST vaccine), control (PBS), Vac (HKST vaccine alone), Al-Vac (alum in combination with the HKST vaccine) and PRP-Vac (PRP in combination with the HKST vaccine) groups. Values are shown as the mean ± SE. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

2.7. Determination of bacterial load in spleens and livers

To evaluate vaccine-induced protective immunity against *S. typhimurium*, an additional experiment was performed to determine the bacterial load in the livers and spleens after challenge with the bacterium. Two weeks after the last immunization, five mice from each group were infected via an intraperitoneal injection with 10^3 live *S. typhimurium* that was suspended in 200 μL of PBS. Forty-eight hours later, the mice were sacrificed and the spleen and liver of each mouse were homogenized individually. Ten microliters of the appropriate dilutions (1:10 in Triton X-100 (0.05%)) of spleen and liver-cell suspensions were then plated separately on trypticase soy plates. One day after culturing at 37 °C, the log of colony-forming units (CFUs) was determined. The optimal dose of *S. typhimurium* for challenge had been determined by preliminary titration (data not shown).

2.8. Survival rate

Two weeks after the last immunization, 10 mice from each group were challenged with 10^7 live *S. typhimurium*. The survival rate was then monitored for 3 weeks. The lethal dose of *S. typhimurium* had been determined by preliminary titration (data not shown).

2.9. Statistical analysis

The MTT assay, cytokine levels and the bacterial loads in the spleen and liver were analyzed using one-way ANOVA, followed by Tukey's post test. The survival rate was measured using Kaplan–Meier analysis and the log rank test. A *p*-value of 0.05 was considered significant.

3. Results

3.1. Cytokine pattern

As shown in Fig. 1A, the mice immunized with the alum–PRP mixture with the HKST vaccine produced significantly more IFN-γ than mice that received either PBS, the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with PRP. Lymphocytes from the mice vaccinated with HKST with PRP produced larger amounts of IFN-γ than those of the mice that received HKST vaccine with alum or HKST vaccine alone; however, the differences between each group were not statistically significant. The mice immunized with the HKST vaccine with PRP produced significantly more IFN-γ than mice that received PBS. Lymphocytes from the mice immunized with HKST vaccine alone produced more IFN-γ

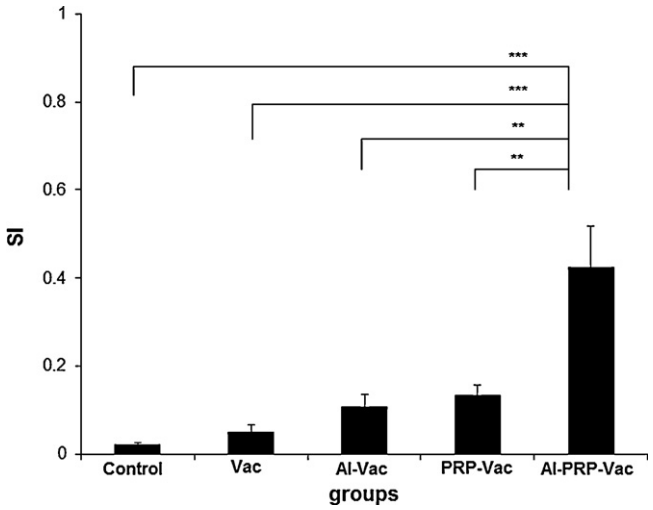

Fig. 2. Effect of administering alum–PRP mixture on lymphocyte proliferation. Two weeks after the final immunization, spleens from individual mice (five per group) were removed and lymphocyte proliferation was evaluated by the MTT method. The groups are as in Fig. 1. Values represent the mean ± SE. SI: Stimulation Index. $^{***}p < 0.001$, $^{**}p < 0.01$.

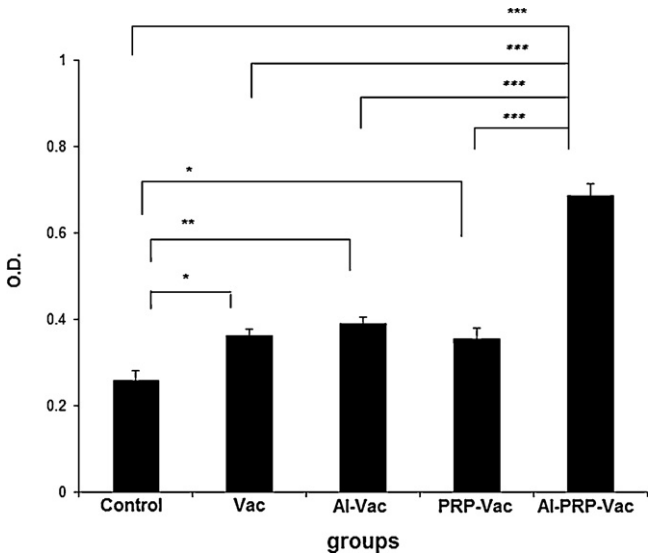

Fig. 3. Effect of administering alum–PRP mixture on IgG antibody response. Two weeks after the final immunization, the sera obtained from individual mice (five per group), were screened for the presence of IgGs against *S. typhimurium*. The groups are as in Fig. 1. Values are mean ± SE of five mice in each group. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

than mice that received PBS or the HKST vaccine with alum. Again, the difference between the groups was not statistically significant.

Mice immunized with alum in combination with the HKST vaccine, produced more IL-5 than mice that received PBS, the HKST vaccine alone, the HKST vaccine with PRP or the HKST vaccine with the alum–PRP mixture; however, the differences between each group were not statistically significant (Fig. 1B). The IL-5 levels were similar among the mice that received HKST vaccine alone or with the alum–PRP mixture. Lymphocytes from the mice that were vaccinated with the HKST vaccine alone or in combination with alum–PRP mixture produced larger amounts of IL-5 compared to the IL-5 levels measured in the mice that received PBS or the HKST vaccine with PRP; but the difference between the groups was not statistically significant.

As shown in Fig. 1C, the IFN-γ/IL-5 ratio from the mice that were immunized with the alum–PRP mixture in combination with HKST was significantly higher than the IFN-γ/IL-5 ratio from mice that were administered PBS, the HKST vaccine alone or the HKST vaccine with alum. The mice that were immunized with HKST vaccine containing the alum–PRP mixture had an increased IFN-γ/IL-5 ratio compared to the mice that received HKST vaccine with PRP; however the differences between the groups were not statistically significant. The mice that received the HKST vaccine with PRP had significantly more IFN-γ/IL-5 ratio than mice that received the HKST vaccine with alum. The mice immunized with the HKST vaccine with PRP had more IFN-γ/IL-5 ratio than mice that received PBS or the HKST vaccine alone; but the differences between the groups were not statistically significant.

3.2. Lymphocyte proliferation

Since lymphocyte proliferation is generally considered to be a measure of cell-mediated immunity, *S. typhimurium*-specific lymphocyte proliferation was evaluated using an MTT assay. As shown in Fig. 2, lymphocyte proliferation was significantly higher in mice treated with the HKST vaccine in combination with the alum–PRP mixture compared to the lymphocyte proliferation in mice that were administered PBS, HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with PRP. Lymphocytes from the mice that were vaccinated with HKST with PRP had more proliferation than the lymphocytes harvested from the mice that received PBS, HKST vaccine alone, the HKST vaccine with alum; however, the differences between each group were not statistically significant. Lymphocyte proliferation of the mice that vaccinated with the HKST vaccine with alum was higher than the mice that received the HKST vaccine alone or PBS; but the difference between the groups was not statistically significant. Lymphocyte proliferation was higher in mice treated with the HKST vaccine alone compared to the lymphocyte proliferation in mice that received the PBS. Again, the difference between the groups was not statistically significant.

3.3. Antibody titer

The sera obtained two weeks after the last immunization, from all the groups of mice, were screened for the presence of IgGs against *S. typhimurium*. As shown in Fig. 3, a significant increase in anti-*S. typhimurium* IgG titers was observed in mice vaccinated with the HKST in combination with the alum–PRP mixture as compared to the mice that were administered PBS, the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with PRP. The mice that received PBS had significantly less anti-*S. typhimurium* IgG titers compared to the IgG titers observed in the mice that were administered the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with PRP. The anti-*S. typhimurium* IgG titers were not significantly different between mice that were vaccinated with the HKST vaccine alone or in combination with alum or PRP.

3.4. IgG isotyping

The isotype profile of antibody responses is related to the cytokines produced by antigen-specific T cells and is an indirect measure of the Th1/Th2 cytokine profile. We determined the relative levels of anti-*S. typhimurium* IgG2a and IgG1 antibodies in the sera obtained two weeks after the last immunization, from all of the groups of mice.

As shown in Fig. 4A, the IgG2a levels were significantly higher in mice that were treated with the HKST vaccine in combination with the alum–PRP mixture compared to those of mice that received PBS, the HKST vaccine alone or the HKST vaccine with alum. Furthermore the IgG2a level was higher in mice treated with the HKST

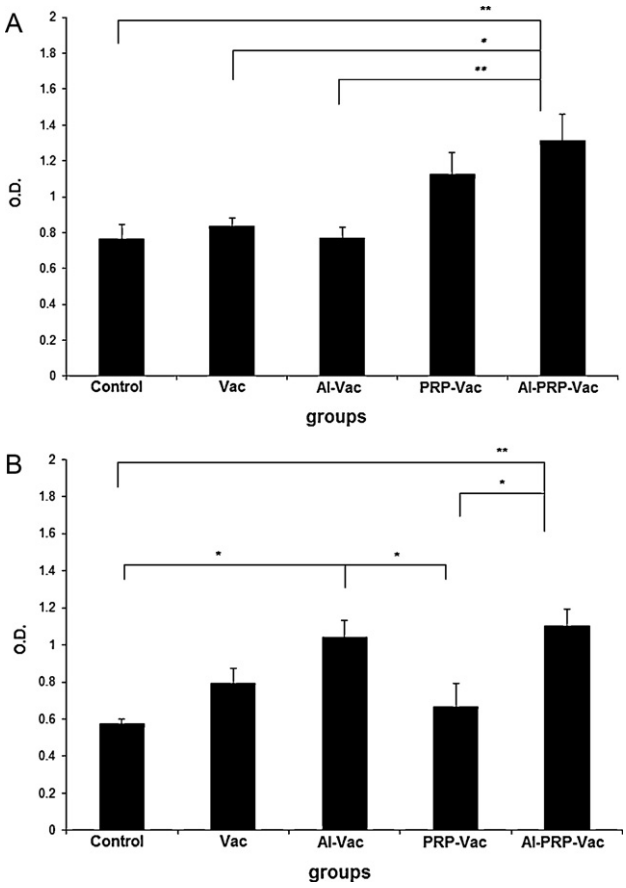

Fig. 4. Effect of administering the alum–PRP mixture on IgG isotyping. The relative levels of anti-*S. typhimurium* IgG2a (A) and IgG1 (B) antibodies in the sera obtained two weeks after the last immunization, from five mice in each group, were determined. The groups are as in Fig. 1. Values are mean ± SE of five mice in each group. $^{**}p < 0.01$, $^{*}p < 0.05$.

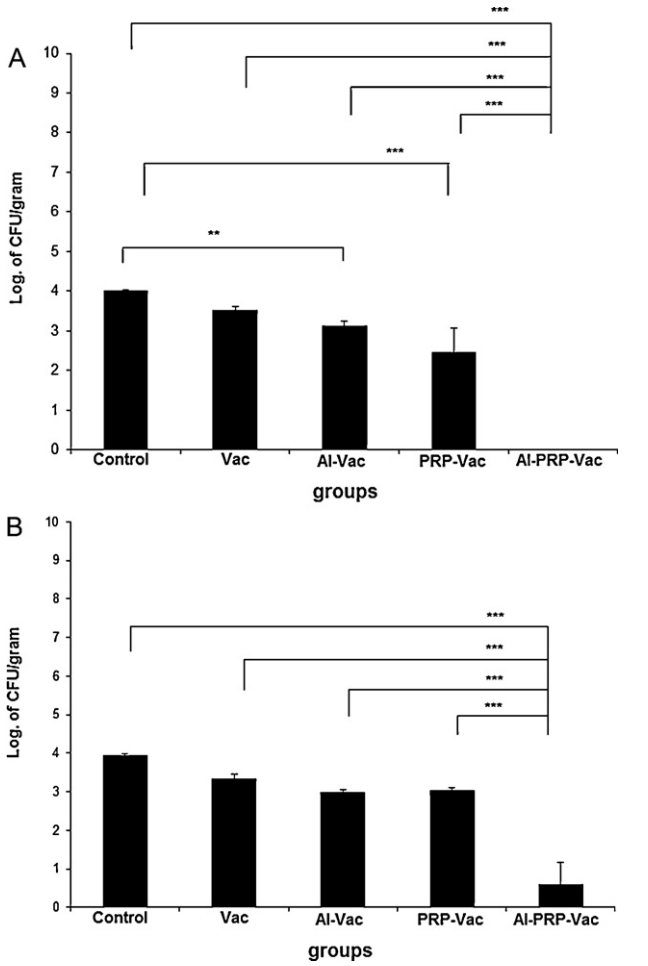

Fig. 5. Bacterial loads in livers (A) and spleens (B) after challenge with live *S. typhimurium*. Two weeks after the last immunization, the mice (five per group) were infected with live *S. typhimurium*. Forty-eight hours post-infection, the liver and spleen from each mouse were homogenized individually and plated on trypticase soy agar plates. One day after culturing at 37 °C, CFUs (log) were determined. The groups are as in Fig. 1. Values represent the mean ± SE. $^{***}p < 0.001$, $^{**}p < 0.01$.

vaccine in combination with the alum–PRP mixture compared to the IgG2a level in mice that were administered the HKST vaccine in combination with PRP; however, the difference was not statistically significant. The mice vaccinated with HKST with PRP had more IgG2a compared to the mice that received PBS, the HKST vaccine alone or the HKST vaccine with alum; but the difference between the groups was not statistically significant.

The mice that were administered HKST vaccine with the alum–PRP mixture or HKST vaccine with alum had significantly more IgG1 compared to the mice that received PBS or HKST vaccine with PRP (Fig. 4B).

3.5. Bacterial load in the spleen and liver

As shown in Fig. 5A, cultures of homogenized livers from the mice that received the HKST vaccine in combination with alum–PRP mixture had significantly fewer mean bacterial colony counts compared to the mean bacterial colony counts observed in mice that received PBS, the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with PRP. The mean liver colony counts from the mice immunized with PRP or alum in combination with HKST vaccine was less than the liver colony counts from the mice that received PBS.

Cultures of homogenized spleens from the mice that were vaccinated with HKST with the alum–PRP mixture had significantly fewer mean bacterial colony counts compared to the mean bacterial colony counts observed from the mice that received PBS, the HKST vaccine alone, the HKST vaccine with alum or the HKST vaccine with PRP (Fig. 5B).

3.6. Survival after S. typhimurium challenge

Two weeks after the second immunization, the survival rates of 10 mice in the groups that were challenged with live *S. typhimurium* were analyzed (Fig. 6). The survival rate of mice which received PBS was significantly less than the survival rates observed in mice that received the vaccine alone or in combination with alum, PRP or alum–PRP mixture. Furthermore, the mice immunized with the HKST vaccine in combination with alum–PRP mixture exhibited a significantly higher survival rate compared to the survival rate of mice that received the HKST vaccine alone. The survival rates of mice that received the HKST vaccine in combination with alum or PRP were higher than the survival rate observed in mice that received the vaccine alone, but the difference between the groups was not statistically significant. The mean survival rate of the mice that were immunized with HKST in combination with alum–PRP mixture was higher than the survival rates of the mice that received the HKST vaccine in combination with alum or PRP; however, the differences were not statistically significant.

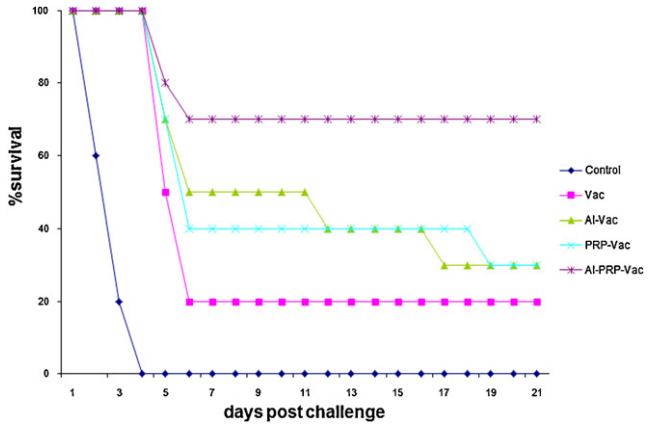

Fig. 6. Survival rates of mice immunized with PBS (control group), HKST vaccine alone (Vac group), alum in combination with the HKST vaccine (Al-Vac group), PRP in combination with the HKST vaccine (PRP-Vac group) or the alum–PRP mixture in combination with the HKST vaccine (Al-PRP-Vac group). Two weeks after the final immunization, the mice were infected with live *S. typhimurium*. Their survival rate was recorded daily for 21 days. Mice in the Vac, Al-Vac, PRP-Vac and Al-PRP-Vac groups showed a significantly higher survival rate than mice in the control group ($p < 0.001$ for all comparisons). While mice in the Al-PRP-Vac group showed a significant higher survival rate than mice in the Vac group ($p < 0.05$), the difference between survival rates of mice in Al-Vac or PRP-Vac group and those of mice in Vac group was not statistically significant. Values are representative of 10 mice per group.

4. Discussion

Here, we investigated the ability of PRP, and the mixture of alum and PRP, to enhance the protection of a HKST vaccine, which functions as a vaccine model against a facultative intracellular bacterium.

Our results showed that the administration of the alum–PRP mixture, when utilized as an adjuvant in combination with the HKST vaccine, significantly increased the vaccine's efficacy. The improved efficacy was associated with increasing IFN-γ production; shift towards a Th1 response (by increasing IFN-γ/IL-5 ratio); lymphocyte proliferation; the production of anti-*S. typhimurium* total IgG, IgG2a and IgG1; and improved resistance and survival against an *S. typhimurium* challenge. The adjuvant activity of the alum–PRP mixture was more than the adjuvant activity of either alum or PRP alone and is comparable with that of naloxone and alum–naloxone mixture which previously had been shown by us [14,16,17,15]. These results indicated that the alum–PRP mixture stimulated both humoral and cellular immune responses. As mentioned above, the most effective immune response against many pathogens, even for some obligate intracellular pathogens, is an immune response that combines both humoral and cellular components [3,4]. The effects of the alum–PRP mixture on cellular immune and humoral responses are more likely due to PRP and alum, respectively.

Immunostimulatory effects of PRP observed in the current study are in line with the finding that treatment of mice with nadolol and ICI118,551, pan beta- and beta2-blockers, respectively, throughout the course of Influenza A virus infection, enhance antiviral TCD8 responses [23]. The immunostimulatory effects due to beta-adrenergic receptor blockers observed in previous studies could be due to the direct influence of these agents on innate and adaptive immune responses, perhaps by binding to innate immune cells and lymphocytes [12]. However, considering the half-life of PRP [24,25], the stimulatory effect of PRP on the immune response observed in the current study may only be due to the effects of PRP on the innate immune response, which in turn influences adaptive immunity. Ultimately, the latter would be triggered by PRP indirectly, without the binding of PRP to its receptors on effector T and/or B lymphocytes.

One possible mechanism of PRP action is to provide a pro-inflammatory milieu by blocking beta2-adrenergic receptors. This inhibition would accelerate local inflammation via inhibiting the effects of APCs-derived catecholamines or catecholamines from other sources such as sympathetic nerves or adrenal glands [26–30].

One of the other possible mechanisms for the adjuvant activity of PRP is the inhibition of regulatory T lymphocytes [31,32]. The inhibitory effect of administration of PRP on regulatory T cells has been shown previously [33]. Furthermore it has been suggested that regulatory T lymphocytes can inhibit dendritic cell (DC) maturation and the expression of costimulatory molecules. This in turn reduces ability of DCs to activate T cells [34–36]. PRP-induced inhibition of the interaction of regulatory T lymphocytes with DCs may enhance vaccine-induced immune responses. This mechanism is in line with findings indicating that administration of CCR4 antagonist as adjuvant accelerates vaccine-induced immunity by inhibition of regulatory T lymphocytes [37,38].

Administration of PRP as an adjuvant, either alone or in combination with alum, may activate APCs via the above-mentioned mechanisms. This would result in the presentation of HKST antigens by activated APCs. However, due to the half-life of PRP [24,25], there is probably little or no PRP in the environment during antigen presentation. Therefore, it is possible that previously activated APCs induce the Th1 and pro-inflammatory immune responses.

The finding of the current study about adjuvant activity of propranolol and our previous studies about adjuvant activity of naloxone [14,16,17,15] emphasizes that the local microenvironment at the time of uptaking and processing an antigen by APCs has a crucial role in the fate of subsequent acquired immune response against the antigen [39,40].

In conclusion, the administration of the alum–PRP mixture as an adjuvant in combination with a HKST vaccine can enhance cellular and humoral immunity and shift the immune response to Th1. As both PRP and alum are approved for human use [41,42], the combined mixture may provide a new, relatively safe means of eliciting effective vaccine-induced Th1 immune responses to malignancies and microbes. Furthermore, our results indicate that administering PRP, without alum, with the HKST vaccine increased cell mediated immunity and shifted immune responses to Th1. To our knowledge, this study is the first one in the literature to evaluate the adjuvant activity of a beta-adrenergic antagonist alone or as a mixture with alum for use in combination with a vaccine. Therefore, follow-up studies are needed to confirm these results and to examine adjuvant activity of PRP and alum–PRP mixture when combined with vaccines against other microbes.

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Conflict of interest: There is no financial and commercial conflict of interest.

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