

## Improved Alum Containing Adjuvant by Vitamin A for Enhancing Immune Responses and Efficacy of *Leptospira* Vaccine in Hamster Model

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### Abstract

Use of suitable adjuvant is one of the priorities in vaccine and immune modulation, stimulation and potentiating. Vaccination is effective for prevention and treatment of bacterial diseases including Leptospirosis. In the present study, we prepared Leptospiral vaccine with Alum, modified Alum adjuvant (mAlum) and without adjuvant. Vaccination was done; then we evaluated the immune responses by isolating the splenocytes and sera for interleukin (IL) profiles (the highest level of all cytokines except IL-4 and IL-12 was obtained in the mAlum antigen group at week 7 post-treatment). Moreover, the expression of all evaluated cytokines in the mAlum group was greater than in the other groups at week 62. Significant increases in antibody titers were noted in the mAlum and Alum group, challenged interaperitionally with a lethal dose of virulent and monitored pathological lesion that moderate to severe lesions with score 3 were observed in the control group while the animals immunized with mAlum-antigen and Alum-antigen displayed slight to mild lesions with an average score of 0.5. The results demonstrated that modified Alum Adjuvants are better than Alum adjuvants as revealed by the enhanced long-term antibody responses. None of the vaccinated animals died from the challenge experiment 84 day post-injection except those vaccinated with saline vaccine. We assume that these observations affirmatively assign a pivotal vitamin a role to adjuvant formulation and preparation in type and extent of immune responses raised in the vaccinated animals.

**Keywords:** Vaccine; Adjuvant; Immunomodulation; Cytokine; Vitamin A

### Introduction

Vaccination as an effective way for prevention and treatment of bacterial diseases like leptospirosis has been widely applied over the last century [1]. Leptospirosis is a zoonotic disease in the developed countries. Many problems following leptospirosis occur in the livestock industry, tourism and sport. Despite enormous efforts to eradicate the disease, more than 500,000 cases are reported each year. Vaccines against leptospirosis were published in 1916 [2]. Since then; whole cell vaccines have been used in humans, cattle, swine, and dogs. However, this type of vaccine has many problems, especially ineffectiveness [1]. In recent year, considerable attempts have been made for improvement of vaccine industry to increase the immune duration, safety and prophylaxis. Induction of the favourable immune response is the ideal goal; however, it is hard to obtain because of the wide varieties broad relationships between immune cells, cytokines, hormones and the nervous system [3]. Accordingly, as noted Kool et al., a more complete understanding of the mechanisms for immunopotential/regulation/modulation and safety is necessary [2]. The immune system regulation is highly depended on the balance between signalling proteins and molecule. Proinflammatory cytokines such as (INF)- $\delta$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin IL-1, IL-6, and IL-12 not only regulate protective immunity against pathogens, but also are involved in induction of some immunopathologies [4,5]. Several of these biologic

effects are also evident during host responses to *leptospira* [6,7]. For example, increased levels of TNF $\alpha$ , IL-12 and IFN- $\delta$  were detected in patients with acute several leptospirosis [3]. It was also seen that IFN- $\delta$  could be responsible for a protective role in hosts (*i.e.*, bovine) that had been previously vaccinated with heat killed *L.borgpetersenii serovar Harjo* [8]. In general, choosing an appropriate adjuvant for stimulation of the innate immune system and preservation of antigens from degradation is important. As part of their effect on host responses to specific antigens, adjuvants have a capability to stimulate release of various TH1 and TH2 cytokines and, ultimately in most cases, humeral responses.

Previous studies have implicated that some components, such as vitamins, could be used for increasing of adjuvants ability [9]. Vitamins could play essential roles in regulating and modulating a broad range of immune functions, such as lymphocyte activation and differentiation, tissue-specific lymphocyte homing and production of specific antibody isotypes. Currently, there are some adjuvants which have been formulated by Vitamin E [9-11]. In this study, different formulation of adjuvants with *Leptospira* vaccine have been studied for their ability on the induction of cellular and humeral immune responses ideal goals were to gain the maximum immune duration, immunomodulatory effects, and affinity of antibody, lymphoprolifration assay, interleukin pattern, and prophylaxis prior challenges.

Materials and Methods

### Leptospira and adjuvant preparation

The three different *Leptospira* serovar including *L.serovars (sejroe hardjo, canicola and grippotyphosa)* were obtained from Razi vaccine and serum research institute (RVSRI), Karaj, Iran [3].

The bacteria were grown in a low protein chemical medium (Banihashemi et al.) suitable for *Leptospira* cultivation. Specifically, the three serovar including were cultured in the media at 27°C for 72 hr and then inactivated using 0.4% formalin. All the material utilized in the medium was obtained from Merck Company.

For uses in these studies, adjuvant comprised of 2.5% AL (OH)<sub>3</sub> aluminium hydroxide gel produced by ALCL<sub>3</sub> (2.5%) and KHPO<sub>4</sub> mg, NaCl (15.6 mg), sodium acetate, and Each ml contains 500,000 IU of vitamin A and the physical conditions by phosphate group and decreasing the affinity antigens conjugated to the Adjuvant and standard release antigen in serum. Each adjuvants was combined separately with a sample of mixed bacterial suspension (at a final concentration 3 × 10<sup>5</sup>)

### Animals

For this study, 90 healthy Syrian hamsters (female 4-wk-old) were purchased from the animal production department of Razi institute. Upon arrive, all were housed under pathogen-free condition in facilities maintained at 27°C with a 45% relative humidity and a 12 hr light dark cycle. All animals had ad libitum access to standard hamster chow and filtered water. All experiments were conducted according to protocols approved by the animal care and use committee (IACUC) of the Razi vaccine serum institute [3,4].

### Vaccination

Prior these studies a pilot safety trial was performed with all the vaccines. For this purpose, 5 hamsters (as well as 5 mice, 5 rabbits, and guinea pigs) were injected with 0.5 ml vaccine intraperitoneally (IP) and subcutaneously (SC). All animals were monitored for anaphylactic shock or significant local inflammation in 14 days after injection [3,4]. The hamsters were randomly allocated in two groups as: experimental (N=60) and control (N=30) groups. Hamsters were SC inoculated with the leptospiral vaccine.

Preparations and then injected with no adjuvant only saline normal (N=20) Alum (N=20), or modified Alum (N=20). Control received either saline or nothing (N=10) or were mock-vaccine-injected and then received Alum (N=10) or mAlum alone (n=10). The vaccinated animals were subjected to booster inoculations at weeks 2 and 62. They were immunized subcutaneously with 1 ml of each vaccine, and boosted 2 times. The first injection was after two weeks, and the second time was on the 62<sup>th</sup> week with the same dose. The animals were weekly bled through the saphenous vein until the week 65. To investigate the immune duration, blood collections were conducted at weeks 3, 6, 9, 12, 15, 64 and 65. All serum samples generated from the collected materials were kept at -80°C prior to use in experimental evaluation (Table 1) [3]. The antibody responses against various vaccine preparations were evaluated by the enzyme-linked immunosorbent assay according to Banihashemi et al. [3].

### Ex vivo lymphoproliferation assay

Lymphoproliferation response was evaluated against the recall antigen. Briefly all animals were euthanized by ketamin and subjected to splenectomy [5,7,12]. The red blood cells (RBCs) were lysed using

red blood cell lysing solution (TAKARA), washed twice in HBSS, suspended in cRPMI (FLUKA), the final cell suspension was counted and viability of the cells assessed using trypan blue. To assess viability, 5 × 10<sup>5</sup> splenocyte was seeded in 96-well plates per each well in 200 µl of complete RPMI. The seeded splenocytes were stimulated by antigens (serotype canicola) for 72 h at 37°C with 5% CO<sub>2</sub>. Phytohemagglutinin PHA (10 µg/ml) and medium were used as positive and negative controls, respectively. DNA synthesis in the stimulated and control cells was measured by ELISA using BrdU colorimetric kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol.

Group	Vaccine	Adjuvant	NO	Booster1	Booster 2	Ch1
1	<i>Leptospira</i> vaccine	--	20	2 weeks	62 weeks	7weeks
2	<i>Leptospira</i> vaccine	Alum	20	2 weeks	62 weeks	7 weeks
3	<i>Leptospira</i> vaccine	mAlum	20	2 weeks	62 weeks	7 weeks
4	--	Alum	10	2 weeks	62 weeks	7 weeks
5	--	mAlum	10	2 weeks	62 weeks	7 weeks
6	Saline Normal	--	10	2 weeks	62 weeks	7 weeks

**Table 1:** Animal immunization schedule.

### Cytokine analysis by quantitative real-time PCR

Total RNA isolation was performed by high pure RNA extraction Kit (Roche, Germany), cDNA synthesis was done using revert aid first strand cDNA synthesis Kit (Fermentas) and real-time quantitative RT-PCR was performed according to manual SYBR premix ex Taq II (Ti RNaseH Plus), bulk kit (TakaRa) [5,6,13]. The primers used of these analyses are presented in Table 2. The PCR cycle was 5 min at 95°C, followed by 45 cycles at 95°C for 30 s and 60°C for 30 s and 75°C for 12 s. Quantization has used the comparative cycle threshold (CT) method by REST software, and reported as relative transcription or the n-fold difference relative to the house keeping gene hypoxanthine phosphoribosyl transferase (HPRT) and beta Actin.

Name	Slain Vaccine	mAlum Vaccine	Alum Vaccine
TNFα	1.26	9.11	1.86
IFNγ	0.51	3.92	1.36
IL12p40	0.47	2.14	1.24
IL4	0.52	1.82	1.11
IL10	0.44	8.41	4.24

**Table 2:** Expression of cytokine mRNA after immunization with antigen plus various.

### Challenge

The hamsters were challenged by intraperitoneal injection in day 49 with 10X MLD<sub>50</sub> (modified LD<sub>50</sub>) of a single passage of *L. interrogans* serovar conicola (Razi Institute), isolated from the kidney of a hamster

inoculated with an isogonics strain [8,12,14]. Survived animals after were challenged on day 97 (55 days after challenge), and the blood samples were collected from the cardiac puncture. The tissues of the infected animals were collected aseptically for histopathology.

## Histopathology

After euthanizing of the animals, samples of the liver, kidney and lung were collected and kept at 10% buffered formalin. The tissues were processed to paraffin blocks, sectioned at 5 µm, deparaffinised, stained with H&E, and finally, examined by a light microscope (NIKON, 80i). Severity of the lesions was studied by an expert pathologist. Quantify classification approach was used to evaluate the severity of the injuries. Tubulointerstitial nephritis was assessed as below: 0=normal, 1=mild, 2=moderate and 3=severe. Liver and lung pathology was graded based on the average of inflammatory foci in 10 × 10 fields: 0=normal, 1=1-3, 2=4-7 and 3 ≥ 7. The extent of pulmonary haemorrhage was graded as 0=none, 1=single focus, 2=multiple foci, and 3=locally extensive (Table 3).

Vaccine and Adjuvants	Average score after weeks 7
vaccine+mAlum	0.3
vaccine+Alum	0.5
vaccine+Saline	1.3
control	3

**Table 3:** Averages of pathological scores in three organs including kidney, liver and lung after weeks 7.

## Statistical analyses

All data are reported as mean ± SD For the cytokine assay, change within groups over time was analysed by the paired t-test. The difference between the vaccinated and control animals was analysed using a one-way ANOVA test. The software used for the statistical analysis was STATA for windows. P<0.05 values were considered statistically significant.

## Results

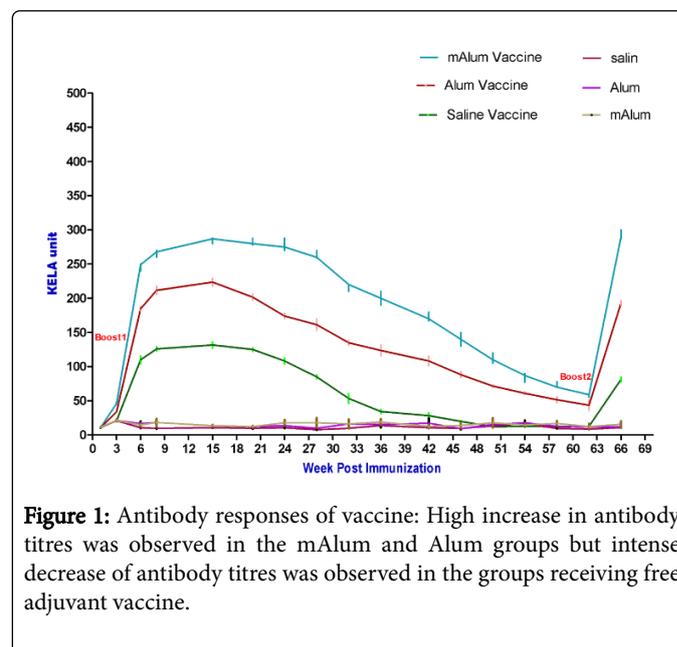
### Characterization of vaccine

During the 14-days of safety test observation period, no anaphylactic shock reaction or significant local inflammations was detected [3].

### Antibody response

The protective efficacy of *Leptospira* vaccine without adjuvant was evaluated in a sensitive hamster through one year. High increase in the antibody titres was observed in the mAlum and Alum group, but an intense decrease of the antibody titres was observed in free adjuvant vaccine receiving groups. Humoral response in the immunized group after one year showed a drastic decrease of antibody titer in the without adjuvant group. These results reveal that protective efficacy of mAlum adjuvant with *Leptospira* vaccine is more than that of Alum adjuvant after one year. To evaluate the strength of a memory cell response, the last booster was injected in week 62. The results after last booster showed that significant increase of antibody titre was belonged

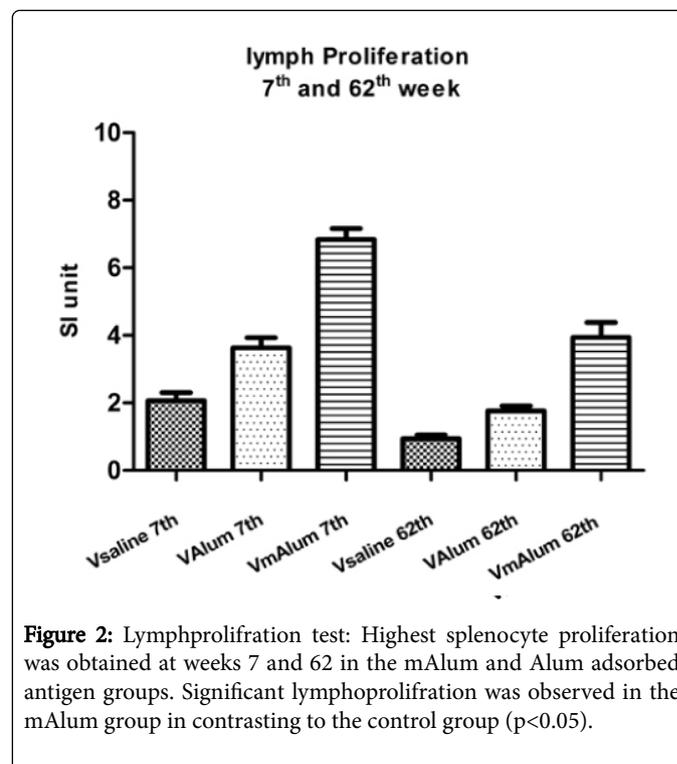
to the mAlum, Alum and without Adjuvant groups, respectively (Figure 1).



**Figure 1:** Antibody responses of vaccine: High increase in antibody titres was observed in the mAlum and Alum groups but intense decrease of antibody titres was observed in the groups receiving free adjuvant vaccine.

### Ex vivo lymphoproliferative response

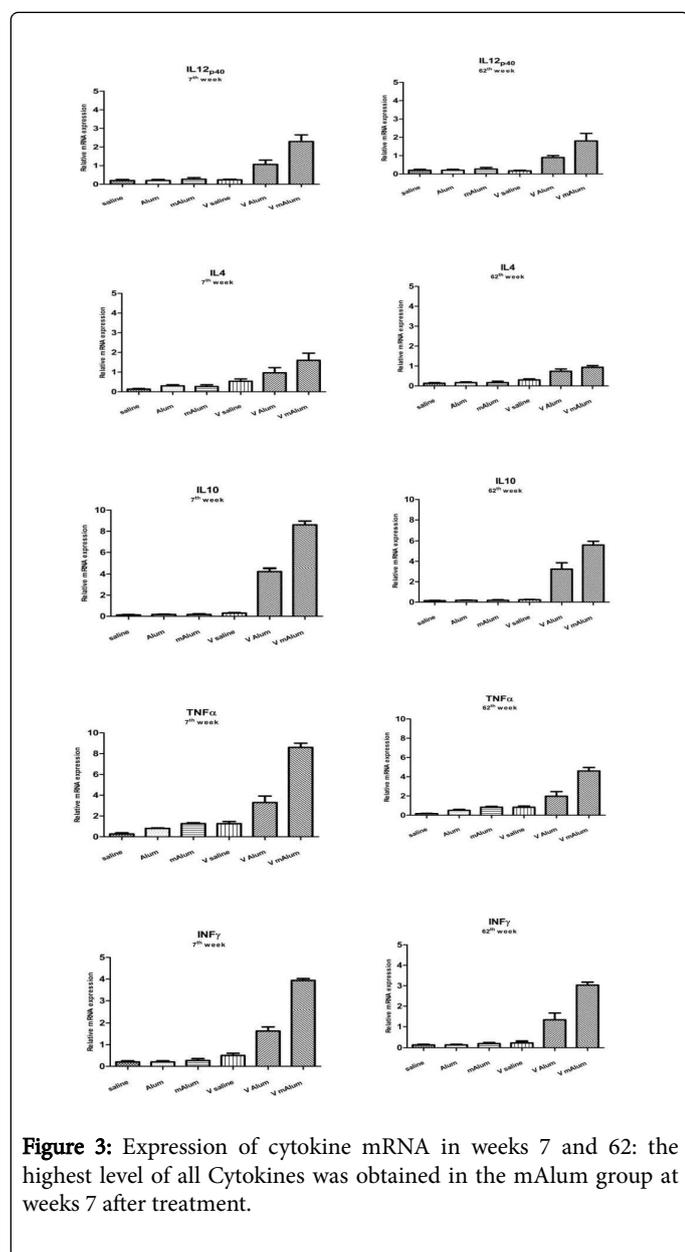
The splenocytes obtained from various groups were subjected to lymphoproliferation assay at weeks 7 and 62 (Figure 2).



**Figure 2:** Lymphoproliferation test: Highest splenocyte proliferation was obtained at weeks 7 and 62 in the mAlum and Alum adsorbed antigen groups. Significant lymphoproliferation was observed in the mAlum group in contrasting to the control group (p<0.05).

*Ex vivo* proliferation after challenging with *Leptospira canicola* without adjuvant was weak compared to the seen with cell from other group, though significant lymphoproliferation was observed contrasting

to the control groups ( $p < 0.05$ ). The greatest proliferation was obtained at week 7 and 62 in mAlum and Alum groups. However, the lymphoproliferation response in the group immunized with mAlum plus antigen was significantly increased in comparison to the Alum and without adjuvant combined antigen groups ( $p < 0.05$ ). Decrease in cell proliferation in weeks 62 was obtained in all groups, and the lowest reduction was observed in the mAlum antigen group. Also lymphoproliferative responses in saline group were similar to those of the control group after 62 weeks. The splenocytes isolated from the animals immunized with PBS-adjuvants failed to show splenocyte proliferation in all groups (Figure 2). Significant splenocyte proliferation occurred in response to PHA (positive control) while groups treated with medium alone could not stimulate cell proliferation (negative control) in both the control and vaccinated groups at weeks 7 and 62.



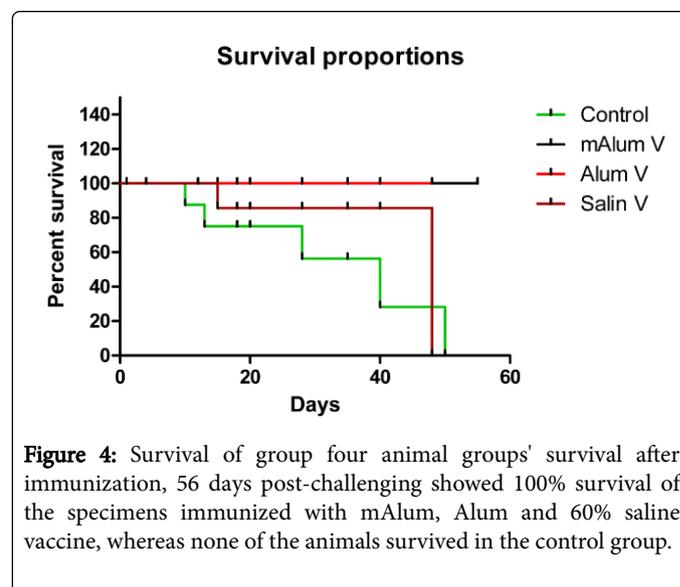
**Figure 3:** Expression of cytokine mRNA in weeks 7 and 62: the highest level of all Cytokines was obtained in the mAlum group at weeks 7 after treatment.

### Cytokine response

The expression of both Th1 (IFN- $\gamma$ , IL-12, TNF- $\alpha$ ) and Th2 (IL-4, IL-10) type cytokines was evaluated from the splenocytes stimulated by antigen with and without various adjuvants using relative quantitative real-time PCR. Based on our findings (Figure 3), the highest level of all cytokines except IL-4 and IL-12 was obtained in the mAlum antigen group at week 7 post-treatment. Moreover, the expression of all evaluated cytokines in the mAlum group was greater than in the other groups at week 62. High levels of Th2 cytokines, especially IL-10, were induced by the mAlum group as compared to other groups with various adjuvants ( $p < 0.05$ ). Apart from Th2 cytokines, mAlum induced a significant Th1 response specified by the elevated levels of TNF- $\alpha$  and IFN- $\gamma$ . The expression of cytokines mRNA from the isolated splenocytes was dropped in all groups at week 62, but the decrease level in the mAlum group was less in comparison to the other groups. Overall, the mAlum group induced a mixed Th1/Th2 response with slight polarization to Th2 response. No significant cytokines mRNA levels of either Th1 or Th2 cytokines were observed in the control group (Figure 3).

### Immunopathology following challenging with various adjuvants-antigens

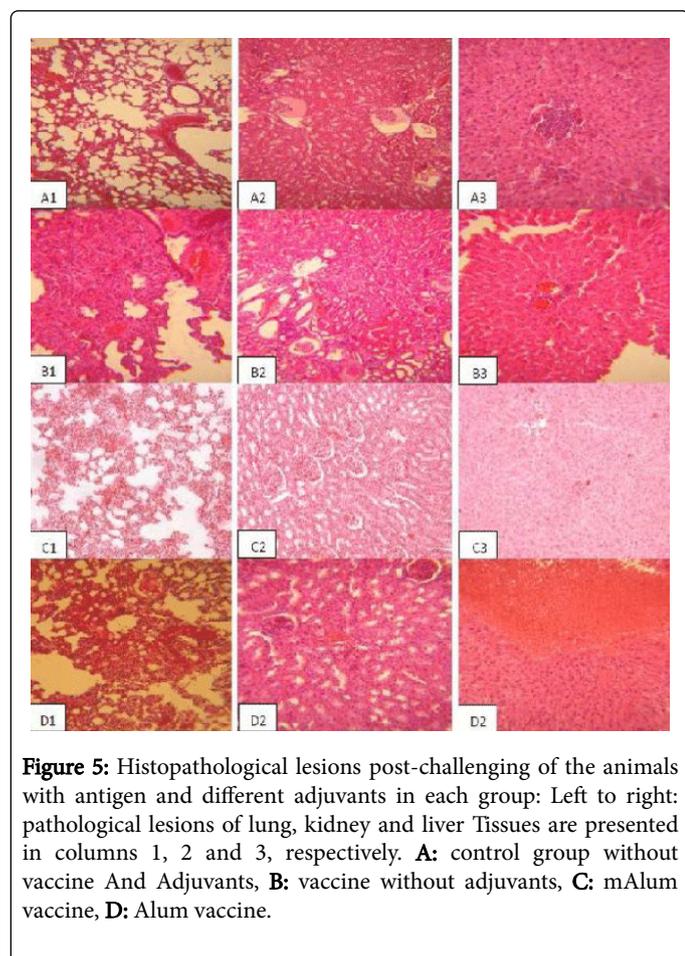
The protective efficacy of antigens delivered through different adjuvants was evaluated in terms of survival and histopathology. Regarding the animal survival after immunization, the results showed 100% survival of the specimens immunized with mAlum, Alum and 60% saline vaccine 56 days post-challenging whereas none of the animals survived in the control group (Figure 4).



**Figure 4:** Survival of group four animal groups' survival after immunization, 56 days post-challenging showed 100% survival of the specimens immunized with mAlum, Alum and 60% saline vaccine, whereas none of the animals survived in the control group.

Mixture of adjuvants with antigens clearly increased the survival rate, and showed significant effects on protection. On the other hand, the histopathological evaluation of various tissues such as kidney, liver and lung in the hamsters (challenged with 10X MLD50 of *Leptospira*) showed alveolar oedema, pulmonary haemorrhage, inflammation, leucocytosis and cell swelling (Figure 5). Pathologic changes at week 7 were scored and are presented in Table 4. Moderate to severe lesions with the score 3 were observed in the control group while the animals immunized with mAlum-antigen and Alum-antigen displayed slight to

mild lesions with an average score of 0.5. Also, immunization with vaccine-saline caused mild lesions in the animals with the score of 1.3.



**Figure 5:** Histopathological lesions post-challenging of the animals with antigen and different adjuvants in each group: Left to right: pathological lesions of lung, kidney and liver Tissues are presented in columns 1, 2 and 3, respectively. **A:** control group without vaccine And Adjuvants, **B:** vaccine without adjuvants, **C:** mAlum vaccine, **D:** Alum vaccine.

## Discussion

Enhancement of the immune response against invading agents by vaccination has been used over the recent century [15]. Leptospirosis is one of the most common zoonotic diseases, and has widespread distribution in the world. By the development of vaccine delivery strategies in recent decades for well induction of immunity and improved patient compliance, the design and development or modification of adjuvants for the immune response alleviation and direction has increasingly become important [16]. In this respect, using the incredible potential of different adjuvants such as modified aluminium hydroxide and oil adjuvants is among the best pioneer options. So, selection of the best combination of vaccine plus adjuvants is necessary [17].

New discoveries have revealed that vitamin A is a key regulator of immune responses. This vitamin has a different role in the immune system [18]. Vitamin A enhances DC Maturation and antigen-presenting capacity by RXR Receptors. This effect has been started to promote T-helper-2 (TH2)-cell differentiation. Furthermore, vitamin A in the presence of transforming growth factor- $\beta$  (TGF $\beta$ ) blocks the differentiation of T helper 17 (TH17) cells, at last give rise fork head box protein 3 (FOXP3)+regulatory T (TReg) cells and down regulating receptor-related orphan receptor- $\gamma$ t (ROR $\gamma$ t) [19,20].

NO	Oligo name	Sequence (5-3)	Tm	CG content	Gene bank accession number
1	TNF- $\alpha$ forward	AACGGCATGTCTCTCAA	50.4	47.10%	AF046215
2	TNF- $\alpha$ reverse	AGTCGGTCACCTTTCT	49.2	50%	
3	IFN- $\gamma$ forward	GACAACCAGGCCATCC	54.3	62.50%	AF034482
4	IFN- $\gamma$ reverse	CAAAACAGCACCGACT	49.2	50%	
5	IL10-forward	TGGACAACATACTACTCACTG	55.9	42.90%	AF046210
6	IL10-reverse	GATGTCAAATTCATTCATGGC	54	38.10%	
7	$\beta$ -actin forward	TCTACAACGAGCTGCG	51.7	56.10%	AF046210
8	$\beta$ -actin reverse	CAATTCCTCTCGGC	51.7	56.30%	
9	IL4-forward	ATCCTGCTCTGCCTTCTAGCATGT	62.7	50%	AF046213
10	IL4-reverse	TTCTTCAAGCACAGGGT CACCTCA	62.7	50%	
11	IL12p40-forward	CTTCCTGAAATGCGAGG CAGCAA	62.7	50%	AF046211
12	IL12p40-reverse	AGCTACTGCTGCTCTTG ACGTTGA	62.7	50%	
13	HPRT-forward	ACATTATGGCCCTCTTGT GTGCTGA	62.7	50%	AF047041
14	HPRT-reverse	GGTTGTAAGTTTGACAA AGGAAAGC	62.7	50%	

**Table 4:** Primers used in this study, Tm and GC content.

Other key topics to be discussed in it are Absorbing power [21,22]. Enclosed antigens in adjuvants are more slowly delivered from the injection site; however, kinetics is mostly dependent on the intensity of adsorption. It is commonly thought that adsorption of antigens to adjuvants is definitive to the effects of adjuvants [3,23]. The maintenance of antigens at the injection site authorizes time for inflammatory cells and antigen-presenting cells to collect at the injection site and interact with vaccines [24,25]. What we know is that regulation and stimulation of the immune system by Alum are independent TLR.

Different methods such as cell damage, physicochemical parameter, cathepsin B, NALP3, IL1 $\beta$ , IL18, PGE2, and interaction of CD80/86 with CD28 have been proposed for activation of the immune system by adjuvants [2,26]. The majority of the researchers, who have studied the Leptospirosis inflammation, have found that *Leptospira* can induce a remarkable increase in the human inflammatory cell and expression of IFN- $\gamma$ , TNF- $\alpha$  and IL-12 receptor [27].

The success, find combination of mAlum-vaccine refers to its capability to promote both innate and adaptive immune responses. On the other hand, it has the ability to direct activation of DCs and rise of

MHC II and CD flowing the challenge of human monocytes with tetanus toxoid and Alum adjuvants [14,28]. By this method, the antigen joined to the surface of Alum (can be slowly delivered) and vitamin A (activation of DC and rise MHC class 2) we could access consistent potent antibody response good [29].

Lympho-proliferative response tests showed that mAlum Adjuvants caused the highest proliferative reaction compared to other adjuvants. Our results indicated that both mAlum and Alum induced noticeably higher level and long-term humoral responses (at week 64 post-treatment). Also, efficient maintenance a very strong Th1-type immune response was impelled following vaccination with an inactivated bacterial vaccine with aluminum hydroxide adjuvant, and the contention of  $\gamma\delta$  T cells in the cellular response was observed [10,30]. In this study, we evaluated Th1 responses, by changing some physicochemical parameters and adding Vitamin A. Moreover, induction of Th1-type cellular immune response is correlated with the protection generated by the bovine *Leptospira* vaccine against *L. borgpetersenii* serovar hardjo. These data, establish that mAlum adjuvants can stimulate the production of type I cytokines involved in cellular immunity [9,10,31]. We know that expression of cytokines is done at different times. In the challenged of hamster model, after vaccination, TNF- $\alpha$ , IFN- $\gamma$  and IL-12 were expressed about 1 hour post-vaccination whereas IL-4 and IL-10 were prominent 1 to 2 days post-challenge [3,32].

The pathological lesions like necrosis and fibrosis seem to be due to the effects of inflammatory reactions [25,33]. Cytokines have been proposed to regulate tissue damage in acute lung injury and pulmonary fibrosis, and similar mechanism of tissue damage and errant repair may also occur in renal tissue infection with *Leptospira*. Furthermore, protection due to the immune response induced in the hamsters after getting mAlum showed prolonged survived rats, and reduced severity of pathological lesions compared to the Alum and without adjuvant. In conclusion, modified Alum adjuvants with whole *Leptospira* induced an effective and long-term immune response and maintenance protection, and could be considered as an appropriate vaccine prevention and therapy strategy provided that all physicochemical parameters and vitamin A that influence the immune responses take into consideration [11,34].

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## Declaration of Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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