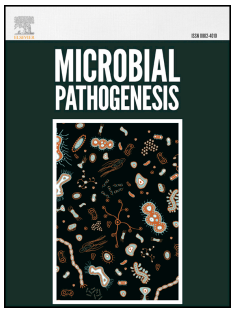

Apoptotic blebs from *Leishmania major*-infected macrophages as a new approach for cutaneous leishmaniasis vaccination

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Apoptotic blebs from *Leishmania major*-infected macrophages as a new approach for cutaneous leishmaniasis vaccination

Running title:

Apoptotic blebs and cutaneous leishmaniasis vaccination

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Abstract

We focused on apoptotic blebs from *Leishmania major*-infected macrophages as a vaccine for cutaneous leishmaniasis. Apoptosis was induced in *Leishmania major*-infected J774A.1 cells in order to prepare apoptotic blebs. Test groups of BALB/c mice were immunized with these at doses of 1×10^6, 5×10^6 or 1×10^7 blebs. An immunization control group received *Leishmania* lysate antigens. The results showed that as the number of apoptotic bodies increased, the lymphocyte proliferation index increased, and this was proportional to IFN-γ level in the test groups. Additionally, the difference of IFN-γ, IL-4, IFN-γ/IL-4 ratio, or total IgG ($p < 0.0001$) in all groups was statistically significant compared to the negative control group. The highest IFN-γ (514.0 ± 40.92 pg/mL) and IFN-γ/IL-4 ratio (2.94 ± 0.22) were observed in the group that received 1×10^7 apoptotic blebs. The highest levels of IL-4 (244.6 ± 38.8 pg/mL) and total IgG (5626 ± 377 μg/mL) were observed in the immunization control group. Reflecting these data, no lesions were observed in any of the groups vaccinated with apoptotic blebs after 12 weeks. In summary, the use of apoptotic blebs from *Leishmania major*-infected macrophages is protective against the challenge with *Leishmania major* in this animal model.

Keywords

Apoptotic bleb; Cutaneous leishmaniasis; *Leishmania major*; Vaccination

1. Introduction

The Neglected Tropical Disease (NTD) leishmaniasis is endemic in 98 countries affecting approximately 12 million people per year with 350 million people living at risk of disease (World Health Organization). Causative *Leishmania* species are sand fly borne kinetoplastid parasitic protozoa [1] and infection can lead to a wide spectrum of clinical pathologies, from self-healing but scarring cutaneous leishmaniasis (CL) to fatal visceral disease (VL). Amongst other factors, this diversity of disease is dependent on the parasite species, host immunity, and genetic background [2]. Largely due to elimination efforts in south Asia, the global burden of VL has decreased substantially in the past decade. However, due to forced migration, the cases of CL have substantially increased in the same period [3]. Approximately, 75% of reported CL cases occur in 10 countries with an incidence rate of 0.7-1.3 million cases annually. However, the actual prevalence of this disease is estimated to be 6 to 10 times higher [4, 5].

Controlling reservoirs and vectors, providing diagnostics and treatment, and the emergence of drug-resistant species are serious challenges for the control of CL. These issues illustrate the need for new drugs for CL or effective vaccines [6-8]. There are three generations of vaccine used against leishmaniasis, 11 of which entered in clinical trials. These include Leishvaccine, ALM, Leishmune, CaniLeish, and GALM for first generation, LEISH-F1, LEISH-F2, LEISH-F3, Leish-Tec, and $SMT^{\gamma}+NH^{\mu}$ for second generation, and ChAd63-KH for third generation. Among these, Leishmune®, CaniLeish®, and Leish-Tec were approved to be used as vaccine in animal [9]. Therefore, there is still no appropriate vaccine available for human use.

In Iran in the 1970s and 1980s vaccination against CL widely used intradermal inoculation of live promastigotes of *L. major* ($2\text{-}3 \times 10^5$), leishmanization. However, this practice was

discontinued due to drawbacks such as ulcer development in a few susceptible individuals [10]. Immunity on leishmanization has been proposed to be due to vaccinated people harboring live parasites in their skin which release excretory/secretory antigens (ESAs), stimulating the host immune system and induce protection [11]. Accordingly, researchers have focused on *Leishmania* spp. ESAs as vaccine targets [12]. However, it is now known that the protective immunity against *L. major* is related to immune system memory, not the parasite presence in the skin tissue [13, 14]. In light of this, studies have focused on several types of vaccine, including live or live-attenuated parasites, the whole killed parasites, *Leishmania* spp. antigens and naked DNA-encoding parasite antigens. However, none so far have had the efficacy to be developed for use as a vaccine in humans [15, 16]. Against this backdrop, we focused on a new approach to vaccination against CL: apoptotic blebs from *Leishmania major*-infected macrophages.

Apoptotic blebs/bodies contain phosphatidylserine and phosphorylcholine on their surface, facilitating the clearance of these bodies by antigen presenting cells such as macrophages [17]. After phagocytosis of an apoptotic bleb, the antigen presenting cells process and present antigenic epitopes to the adaptive immune cells on class I and class II major histocompatibility complex (MHC-I and -II) molecule. However, there is preferential antigen presentation on MHC-I and therefore the cell-mediated immune response in stimulated, this is important for an effective CL vaccine [18-21]. Therefore, in this study we evaluated apoptotic blebs from *Leishmania major*-infected macrophages as a vaccine candidate for CL in a murine model.

2. Materials and Methods

2.1. *Leishmania* maintenance

Leishmania major (MRHO/IR/75/ER) was used in the present study. Promastigotes were cultured and maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented by fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and penicillin-streptomycin solution (pen-strep; 100 IU/mL penicillin and 100 µg/mL streptomycin; Sigma-Aldrich, Missouri, USA). In order to reduce the proliferation of the parasite and to maintain it without passage for about 2 months, the amount of FBS was adjusted to 2% and the number of parasites to 10,000/mL. In addition, the culture medium containing the parasite was incubated at 24 °C for 24 hours and then kept at 4 °C for long-term maintenance. Daily monitoring of the growth of the parasite and the absence of fungal and bacterial contamination was undertaken [22].

2.2. Animal model

8-10 week-old female BALB/c mice (Pasteur Institute, Tehran, Iran), 20-25 gram, were utilized. The project underwent ethical review and was approved by the Ethics Committee of Mazandaran University of Medical Sciences (Code: IR.MAZUMS.REC.1397.315). The care and use of experimental animals complied with local animal welfare laws, guidelines, and policies.

2.3. Apoptosis induction in *Leishmania major*-infected macrophages

The cell line macrophages (J774A.1) were cultured in the same medium as used for the parasite culture but with 20% FBS as complete medium. 2×10^6 cells, counted using a Neubauer haemocytometer, were added to each 75 cm^2 cell culture flask (Corning, Oneonta, NY, USA), containing 10 mL of the complete medium. The flasks were incubated at 37 °C, 5% CO_2, and 95% humidity for 5-7 days (80% confluent, approximately 9.4×10^6 cells per flask) and 10 stationary phase promastigotes per macrophage (9.4×10^7) were added. The infection rate of

117 macrophage was calculated as 90.7% to 92.8%. After a further 24 hour incubation, apoptosis was
118 induced in the infected cells using the Apoptosis Inducer Kit (Abcam, Cambridge, UK) according
119 to the manufacturer's instruction. Afterwards, the suspensions obtained were filtered using a 5
120 μm pore size membrane filter (Corning, Oneonta, NY, USA) and some of the solution was
121 cultured and the parasite did not grow in the medium. The solution was centrifuged at 20,000 ×g
122 for 15 minutes, the supernatants were discarded, and 1 mL of normal saline (0.85% w/v NaCl)
123 was added to the precipitate. Following evaluation as below, the suspension of apoptotic blebs
124 was stored at -20 °C until use.
125
126 2.4. Differentiation of apoptotic- from necrotic bodies by flow cytometry
127 The Annexin V-FITC Apoptosis Staining/Detection Kit (Abcam, Cambridge, UK) was used to
128 differentiate apoptotic from necrotic bodies. The reaction mixtures were prepared according to
129 manufacturer's instruction and read using as Coulter® Epics XL-MCL™ Flow Cytometer
130 (Beckman Coulter, Fullerton, CA, USA). Briefly, four situations could occur: intact living cells
131 which do not stain with both annexin V and PI; early apoptotic cells that stain with annexin V but
132 do not stain with PI; late apoptotic cells that stain with both annexin V and PI; and necrotic cells
133 that stain with PI but not annexin V.
134
135 2.5. Apoptotic blebs examination using scanning electron microscope (SEM)
136 The apoptotic bleb-containing solution were affixed to the surface of a metal stub, coated with
137 gold through a SEM sputter coater (Bio-Rad, West Chester, PA, USA) and examined using
138 Hitachi S-4300 SEM (Hitachi Science Systems, Tokyo, Japan) at accelerating voltages between 5
139 and 25 kV.

140

141 2.6. Preparation of *Leishmania* lysate antigens

142 As an immunization control, *Leishmania* lysate antigens (LLAs) were prepared by lysis of *L.*
143 *major* promastigotes. 1×10^9 promastigotes in 5 mL of normal saline were lysed by bead-beating
144 using 0.5 mm diameter glass beads (Sigma, Tokyo, Japan), 2 mL of beads were added to the
145 suspension and this was vortexed vigorously for 5 minutes in 1 minute cycles with 10 seconds on
146 ice between. The resulting lysate was passed through a 0.22 μm pore size membrane filter
147 (Corning, Oneonta, NY, USA), and the protein concentration was measured by the Bradford
148 method [23]. The LLAs solution was stored at -20 °C until use.

149

150 2.7. Immunization of mice

151 55 BALB/c mice were randomly divided into 5 groups of 11, of which three test groups received
152 1×10^6, 5×10^6 or 1×10^7 apoptotic blebs, prepared above, in 0.5 mL of normal saline. One of
153 the two remaining groups was considered as immunization control and received 100 μg of LLAs,
154 the remaining group served as the negative control and received 100 μL of normal saline. The
155 groups were subcutaneously inoculated twice at intervals of 14 days.

156

157 2.8. Cytokine and antibody assay

158 2 weeks after the second injection, approximately 300 μL of blood was taken from the tail of the
159 mice. The samples were centrifuged at 2000 ×g, 4 °C, for 10 minutes, the serum was separated
160 and kept at -20 °C until use. Mouse IFN Gamma PicoKine™ ELISA Kit, Mouse IL-4 PicoKine
161 ™ ELISA Kit, and Mouse Total IgG PicoKine ™ ELISA (BosterBio, Wuhan, China) were used
162 to measure the levels of IFN-γ, IL-4 and total IgG respectively in the serum samples according to

the manufacturer's protocol. The reactions were analyzed using Bio-Rad Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA) and the value from each sample was calculated according to the plotted standard curve provided in the kits.

2.9. Lymphocyte proliferation assay

24 hours after blood sampling, 5 mice from each group were randomly selected and euthanized. Subsequently, 5 ml of normal saline was immediately injected into the peritoneal cavity of each mouse and the leukocytes containing peritoneal fluid were harvested. To isolate lymphocytes from peritoneal macrophages the cell suspension of each mouse was poured into separate 12.5 cm^2 cell culture flasks (Corning, Oneonta, NY, USA) and incubated at 37 °C, 5% CO_2, and 95% humidity for 3 hours. The supernatants, containing lymphocytes, were then harvested and centrifuged at 400 ×g, 4 °C for 10 min. The supernatants were discarded and 1 mL of phenol red free RPMI-1640 with pen-strep and 5% FBS was added to the precipitates. For each mouse 5×10^5 lymphocytes, in 100 μL of media, were added into 9 wells of a 96-well microplate (BrandTech Scientific, Essex, CT, USA). 10 μg of LLAs was added to three wells as the immunization control and the mitogen PHA (2.5 μg/mL) was added to three as the control for normalization [24]. The remaining three wells were considered the negative control and nothing was added. The final volume of each well was adjusted to 150 μL using media. The plates were incubated at 37 °C, 5% CO_2, and 95% humidity for 48 hours. Subsequently, 20 μL of MTT stock solution (5 mg/mL; Gibco, Carlsbad, CA, USA) was added to each well and the volume adjusted to 200 μL using the medium (final concentration of MTT was 0.5 mg/mL). The plates were incubated as above for 4 hours and then centrifuged at 400 ×g for 10 minutes. The supernatants were discarded and 100 μL of acidic dimethyl sulfoxide (DMSO 1% of 1N HCl; Sigma-Aldrich,

Missouri, USA) was added to each well. The wells were rotated gently for 10 minutes using shaking incubator (Eppendorf, Hamburg, Germany), centrifuged at 10,000 ×g for 5 minutes and the supernatants were collected and analyzed using a scanning multiwell spectrophotometer (Biotek, Burlington, VT, USA) at the wavelength of 540 nm. The obtained optical densities (ODs) were analyzed according the following formula:

Firstly, for each mouse the stimulation index (SI) of lymphocytes was calculated for the treated lymphocytes with LLAs and PHA as follows:

SI (LLA): $\frac{\text{mean OD of three LLA wells} - \text{mean OD of three NC wells}}{\text{mean OD of three NC wells}}$

SI (PHA): $\frac{\text{mean OD of three PHA wells} - \text{mean OD of three NC wells}}{\text{mean OD of three NC wells}}$

NC is negative control

Then:

$\Delta SI = SI\ (LLA) - SI\ (PHA)$

The mean ΔSI was calculated for each group of 5 mice.

Finally, the proliferation index (PI) was calculated:

$PI = 2^{(\Delta SI \text{ of the treated group} - \Delta SI \text{ of the NC group})}$

NC is the negative control group (i.e. untreated mouse group); the treated group is each of the mice groups treated with apoptotic blebs or LLA.

PI indicates the potency of lymphocyte proliferation in response to injection of each treated group.

2.10. Infection challenge

The 6 mice which remained in each group were utilized in a challenge experiment [25]. 5×10^5 stationary phase promastigotes in a 10-μL volume were inoculated intradermally 3-4 mm from the base of the tail of the mice. The mice were monitored weekly for 12 weeks for lesion development. The formula used in our study was that for ellipsoid lesions [26]:

$$LS = ([A/2 \times B/2 \times C/2] \times 4/3\pi)$$

Where LS is the lesion size in mm^3; "A" is depth of the lesion in mm; "B" and "C" respectively are the width and length of the lesion in mm.

2.11. Data analyses

Shapiro-Wilk statistical test was used to measure normal distribution of data. Data were analyzed using IBM SPSS v16 software (IBM Corp., Armonk, NY, USA) based on the two-tailed *t*-test and analysis of variance (ANOVA) statistical methods. In addition, *post hoc* comparison was performed between groups using the Tukey's HSD test. The statistical differences were considered significant where p value < 0.05. The ELISA data were analyzed using GraphPad Prism v6 software (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Quantity and quality of apoptotic blebs from *Leishmania major*-infected macrophages

The blebs were quantified using flow cytometry. 86.6% of the bodies identified were apoptotic, of which 30.1% were early apoptotic bodies and 56.5% were late apoptotic bodies. The total number of these bodies was 20,606,700 per mL (Figure 1).

The quality of the apoptotic bodies was established using SEM. The average size was 3.88 μm (95% CI 2.69-5.07), and the bodies were uniform and slightly oval (> 98%) in shape. All had an intact cell membrane (Figure 2).

3.2. Cytokine and antibody assay

IFN-γ, IL-4, and total IgG levels were assessed in the serum samples from the immunized mice. Statistical analyses, using ANOVA, showed that there was a significant difference for the levels of each of total IgG, IFN-γ and IL-4, as well as the IFN-γ/IL-4 ratio, among the groups of mice ($p < 0.0001$) (Table 1). Moreover, the pairwise comparison between the groups is shown in Table 2. In addition, the lowest levels of total IgG (1522 ± 327 μg/mL), IFN-γ (153.1 ± 27.3 pg/mL), IL-4 (135.3 ± 28.5 pg/mL), and the lowest IFN-γ/IL-4 ratio (1.13 ± 0.07) were observed in the negative control group. The highest level of IFN-γ (514.0 ± 40.92 pg/mL) and IFN-γ/IL-4 ratio (2.94 ± 0.22) was observed in the group that received 1×10^7 apoptotic blebs (maximum dose). The highest levels of IL-4 (244.6 ± 38.8 pg/mL) and total IgG (5626 ± 377 μg/mL) were observed in the immunization control group (Figure 3).

3.3. Lymphocyte proliferation assay

The proliferation index of the three groups that received apoptotic blebs and the immunization control LLA group, was calculated relative to the negative control group (ΔSI = -1.1). The lowest proliferation index was observed for the group that received 1×10^6 apoptotic blebs (8.75) and the highest (24.25) was for the group that received 1×10^7 apoptotic blebs (Figure 4). The results of statistical analysis between the groups are shown in Figure 4.

3.4. Lesion size evaluation

No lesion development was observed in any of the groups that received apoptotic blebs. However, lesion development was observed in the mice from the negative and immunization control LLA groups. Maximum lesion size was 11.7 ± 1.03 mm^3 in the negative control group and 5.89 ± 0.19 mm^3 in the immunization control group at the 12th week. The mean ± standard deviation of the lesion size in mm^3 in the mice of the negative and immunization control groups from week 1 to week 12 is shown in Figure 5. There was a statistically significant difference between the negative control group and each of the groups received apoptotic blebs ($p < 0.0001$). Moreover, a significant statistical difference was observed between the immunization control group and each of the groups received apoptotic blebs ($p < 0.001$).

4. Discussion

There are many challenges for the development of a CL vaccine, including the genetic diversity in human populations and in the species and strains of *Leishmania* parasite, the type of vaccine, dose of vaccine, and route of vaccine administration [27]. The purpose of the vaccination is the development of immunological memory, and both $CD4^+$ and $CD8^+$ T cells are important for the immunity against *L. major*. Therefore, it is clear that an effective vaccine should not only be safe and easily available but also should be capable of supporting the prolonged induction of $CD4^+$ and $CD8^+$ T lymphocytes [28].

The lymphocyte proliferation test is a non-specific test used to evaluate the lymphocyte response to specific antigens and is useful to evaluate the efficacy of a vaccine [29, 30]. In the reported study, it was observed that as the number of apoptotic bodies increased, the lymphocyte proliferation index increased and this was proportional to IFN-γ measured in the test groups.

Previously, the lymphocyte proliferation response was used to assess three parasitic antigens as vaccines, the lowest proliferation index was observed in the LLAs injected group and this was consistent with the measured IFN-γ [31]. Therefore, lymphocyte proliferation is an indicator of the cellular immune response, especially IFN-γ production [32].

One component of a vaccine is usually an adjuvant to enhance specific immune responses and increase vaccine efficacy. *L. major* ribosomal protein (LRP) was evaluated as a vaccine alone or in combination with CpG oligodeoxynucleotides (CpG-ODN) as an adjuvant. Injection of the vaccine with the adjuvant (LRP+CpG) significantly increased the IFN-γ response in splenocyte supernatants of the mice whilst considerably decreasing the amount of IL-4 and IL-10, with IFN-γ/IL-10 ratio ~ 40 and IFN-γ/IL-4 ratio ~ 100 [33]. This IFN-γ/IL-4 ratio is much larger than IFN-γ/IL-4 ratio of the group that received 1×10^6 the apoptotic blebs (95% CI 1.37-1.45) in the reported study. Iborra, Parody [33] showed that the immune responses prevented the formation of lesions in the challenged BALB/c mice; however, some swelling was observed in the LRP+CpG vaccinated mice and the few parasites were found in the tissue. In the reported study, no lesions were observed in the challenged mice received apoptotic blebs, precluding the measurement of parasite burden.

Few adjuvants are acceptable for human use, and there are many limitations to introducing a new one [34]. Interestingly, apoptotic blebs can be both a vaccine and an adjuvant in that foreign antigen containing apoptotic bodies act as natural adjuvants that stimulate a specific cellular immune response mediated by $CD8^+$ T cell, enhancing the vaccine efficacy and providing stable immunity [35-37]. The results of the reported study support this approach, however, to evaluate the role of host factors in the induction of immunity observed in this study, future work will need

296 to analyze the effects of apoptotic blebs from uninfected macrophages on the induction of an
297 immune response.

298 LLAs-loaded liposomes have also resulted in an improved, although non-protective, immune
299 response, which increased IFN-γ and reduced IL-4 compared to LLAs alone [38]. The highest
300 amount of total IgG was quantified in the LLAs alone group [38]. Similarly, in the reported
301 study, the highest level of total IgG was observed in the LLAs group, consistent with the high
302 level of IL-4. Furthermore, LLAs have been shown to induce a higher IgG response than
303 recombinant *Leishmania* homolog of receptors for activated C-kinase (rLACK) antigens in
304 BALB/c mice [39]. An increase in the amount of IFN-γ level and a decrease in IL-10 level were
305 also observed in splenocyte supernatants of the rLACK group compared to the LLAs group, with
306 an IFN-γ/IL-10 ratio of 55 for the rLACK group compared to 15.3 for LLAs. This indicated more
307 potent cellular immune response to rLACK, however, this response was not protective against *L.*
308 *major* challenge [39]. Similarly, in the reported study, IgG and IL-4 were higher in the LLAs
309 group than in the test groups (i.e. the received apoptotic blebs). However the IFN-γ/IL-4 ratio for
310 rLACK (~ 329) was very much larger than in the apoptotic blebs test groups (1.41-2.94
311 depending on dose), despite protective immunity being observed in the latter but not the former.

312 The quantity of IFN-γ produced on vaccination is considered to be the most important factor
313 in the development of a protective immune response against CL [40, 41]. Interestingly, despite
314 the lack of lesion growth in the groups that received apoptotic blebs, IFN-γ response in the mouse
315 sera was much lower than that obtained in other studies that found no protection against
316 challenge [38, 39]. To evaluate the effect of IFN-γ on preventing lesion development, IL-12
317 together with *L. braziliensis* was inoculated into a murine model and a significant increase in
318 IFN-γ was observed. However, this did not prevent *L. brasiliensis* lesion development [42].

Consequently, it can be concluded that factors other than the quantity of IFN-γ and IL-4, and the IFN-γ/IL-4 ratio, are central to the development of a protective immune response for CL.

Clearly our understanding of IFN-γ and IL-4 function in CL is incomplete, IL-4 and IL-4RA knockout mice are rendered susceptible to challenge with *L. major* [43]. Furthermore, it is expected that during the lesion healing, IL-4 will be reduced and IFN-γ increased, however the expression level IL-4 in the margin of the late lesions was found be to higher than IFN-γ [44].

5. Conclusions

In the presented study, apoptosis was induced in *Leishmania*-infected macrophages and the apoptotic-blebs collected. These bodies will contain fragments of the parasite and together can function as analogous to a whole *Leishmania.* Whilst the mechanism of inducing protective immune response has not been evaluated for the apoptotic-blebs from *Leishmania major*-infected macrophages in the current study, it is likely that these 'fragments' constitute a complex antigenic picture necessary for immunity. The results showed that vaccination with even the lowest number (1×10^6) tested of these apoptotic-blebs, is protective against *L. major* challenge. In addition, we propose that a factor other than the quantity of IFN-γ and the IFN-γ/IL-4 ratio is likely to play a role in providing this protection, this will be the focus of future studies.

Conflicts of interest

The authors declare that there is no conflict of interest.

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References

[1] Stuart K, Brun R, Croft S, Fairlamb A, Gurtler RE, McKerrow J, et al. Kinetoplastids: related protozoan pathogens, different diseases. J Clin Invest. 2008;118:1301-10.

[2] Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. Lancet Infect Dis. 2007;7:581-96.

[3] Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet. 2018;392:951-70.

[4] Akhtari J, Faridnia R, Kalani H, Bastani R, Fakhar M, Rezvan H, et al. Potent in vitro antileishmanial activity of a nanoformulation of cisplatin with carbon nanotubes against Leishmania major. Journal of Global Antimicrobial Resistance. 2019;16:11-6.

[5] Faridnia R, Kalani H, Fakhar M, Akhtari J. Investigating in vitro anti-leishmanial effects of silibinin and silymarin on Leishmania major. Ann Parasitol. 2018;64:29-35.

[6] De Luca PM, Macedo ABB. Cutaneous Leishmaniasis Vaccination: A Matter of Quality. Frontiers in immunology. 2016;7:151-.

[7] Kumar R, Engwerda C. Vaccines to prevent leishmaniasis. Clinical & Translational Immunology. 2014;3:e13.

[8] Mbekeani AJ, Jones RS, Bassas Llorens M, Elliot J, Regnault C, Barrett MP, et al. Mining for natural product antileishmanials in a fungal extract library. Int J Parasitol Drugs Drug Resist. 2019;11:30026-0.

[9] Moafi M, Rezvan H, Sherkat R, Taleban R. Leishmania Vaccines Entered in Clinical Trials: A Review of Literature. International journal of preventive medicine. 2019;10:95-.

[10] McCall L-I, Zhang W-W, Ranasinghe S, Matlashewski G. Leishmanization revisited: Immunization with a naturally attenuated cutaneous Leishmania donovani isolate from Sri Lanka protects against visceral leishmaniasis. Vaccine. 2013;31:1420-5.

[11] Girard-Madoux MJH, Kautz-Neu K, Lorenz B, Ober-Blobaum JL, von Stebut E, Clausen BE. IL-10 signaling in dendritic cells attenuates anti-Leishmania major immunity without affecting protective memory responses: J Invest Dermatol. 2015 Nov;135(11):2890-2894. doi: 10.1038/jid.2015.236. Epub 2015 Jun 22.

[12] Sundar S, Singh B. Identifying vaccine targets for anti-leishmanial vaccine development. Expert review of vaccines. 2014;13:489-505.

[13] Glennie ND, Volk SW, Scott P. Skin-resident CD4+ T cells protect against Leishmania major by recruiting and activating inflammatory monocytes. PLoS Pathog. 2017;13:e1006349.

[14] Zaph C, Uzonna J, Beverley SM, Scott P. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. Nat Med. 2004;10:1104-10.

[15] Iborra S, Solana JC, Requena JM, Soto M. Vaccine candidates against leishmania under current research. Expert Rev Vaccines. 2018;17:323-34.

[16] Launois P, Tacchini-Cottier F, Kieny MP. Cutaneous leishmaniasis: progress towards a vaccine. Expert Rev Vaccines. 2008;7:1277-87.

[17] Peng Y, Martin DA, Kenkel J, Zhang K, Ogden CA, Elkon KB. Innate and adaptive immune response to apoptotic cells. J Autoimmun. 2007;29:303-9.

[18] Rosales C, Uribe-Querol E. Phagocytosis: A Fundamental Process in Immunity. BioMed Research International. 2017;2017:18.

[19] Ramachandra L, Simmons D, Harding CV. MHC molecules and microbial antigen processing in phagosomes. Curr Opin Immunol. 2009;21:98-104.

[20] Espinosa-Cueto P, Magallanes-Puebla A, Castellanos C, Mancilla R. Dendritic cells that phagocytose apoptotic macrophages loaded with mycobacterial antigens activate CD8 T cells via cross-presentation. PLOS ONE. 2017;12:e0182126.

[21] Scott P, Novais FO. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. Nature reviews Immunology. 2016;16:581-92.

[22] Langdon SP. Cell Culture Contamination. In: Langdon SP, editor. Cancer Cell Culture: Methods and Protocols. Totowa, NJ: Humana Press; 2004. p. 309-17.

[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.

[24] Norian R, Delirezh N, Azadmehr A. Evaluation of proliferation and cytokines production by mitogen-stimulated bovine peripheral blood mononuclear cells. Veterinary research forum : an international quarterly journal. 2015;6:265-71.

[25] Charan J, Kantharia ND. How to calculate sample size in animal studies? Journal of pharmacology & pharmacotherapeutics. 2013;4:303-6.

[26] Maurer M, Lopez Kostka S, Siebenhaar F, Moelle K, Metz M, Knop J, et al. Skin mast cells control T cell-dependent host defense in Leishmania major infections. Faseb J. 2006;20:2460-7.

[27] Srivastava S, Shankar P, Mishra J, Singh S. Possibilities and challenges for developing a successful vaccine for leishmaniasis. Parasites & vectors. 2016;9:277.

[28] Hohman LS, Peters NC. CD4+ T Cell-Mediated Immunity against the Phagosomal Pathogen Leishmania: Implications for Vaccination. Trends in Parasitology. 2019;35:423-35.

[29] Maslak PG, Dao T, Krug LM, Chanel S, Korontsvit T, Zakhaleva V, et al. Vaccination with synthetic analog peptides derived from WT1 oncoprotein induces T-cell responses in patients with complete remission from acute myeloid leukemia. Blood. 2010;116:171-9.

[30] Ghaffari G, Passalacqua DJ, Bender BS, Briggs DJ, Goodenow MM, Sleasman JW. Human lymphocyte proliferation responses following primary immunization with rabies vaccine as neoantigen. Clinical and diagnostic laboratory immunology. 2001;8:880-3.

[31] Khezri P, Shahabi S, Abasi E, Hajipirloo HM. Comparison of immunogenical potency of Leishmania major (MRHO/IR/75/ER) antigens prepared by 3 different methods in conjunction with Alum-Naltrexone adjuvant in BALB/c mice. Alexandria Journal of Medicine. 2018;54:503-10.

[32] Suthumchai N, Srinoulprasert Y, Thantiworasit P, Rerknimitr P, Tuchinda P, Chularojanamontri L, et al. The measurement of drug-induced interferon gamma-releasing cells and lymphocyte proliferation in severe cutaneous adverse reactions. J Eur Acad Dermatol Venereol. 2018;32:992-8.

[33] Iborra S, Parody N, Abanades DR, Bonay P, Prates D, Novais FO, et al. Vaccination with the Leishmania major ribosomal proteins plus CpG oligodeoxynucleotides induces protection against experimental cutaneous leishmaniasis in mice. Microbes Infect. 2008;10:1133-41.

[34] Garg R, Babiuk L, van Drunen Littel-van den Hurk S, Gerdts V. A novel combination adjuvant platform for human and animal vaccines. Vaccine. 2017;35:4486-9.

[35] Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature. 1998;392:86-9.

[36] Chattergoon MA, Kim JJ, Yang JS, Robinson TM, Lee DJ, Dentchev T, et al. Targeted antigen delivery to antigen-presenting cells including dendritic cells by engineered Fas-mediated apoptosis. Nat Biotechnol. 2000;18:974-9.

[37] Feng H, Zeng Y, Graner MW, Katsanis E. Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. Blood. 2002;100:4108-15.

[38] Mehravaran A, Nasab MR, Mirahmadi H, Sharifi I, Alijani E, Nikpoor AR, et al. Protection induced by Leishmania Major antigens and the imiquimod adjuvant encapsulated on liposomes in experimental cutaneous leishmaniasis. Infect Genet Evol. 2019;70:27-35.

[39] Lakhal-Naouar I, Koles N, Rao M, Morrison EB, Childs JM, Alving CR, et al. Transcutaneous immunization using SLA or rLACK skews the immune response towards a Th1 profile but fails to protect BALB/c mice against a Leishmania major challenge. Vaccine. 2019;37:516-23.

[40] Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med. 2007;13:843-50.

[41] Kima PE, Soong L. Interferon gamma in leishmaniasis. Front Immunol. 2013;4.

[42] Novais FO, Wong AC, Villareal DO, Beiting DP, Scott P. CD8(+) T Cells Lack Local Signals To Produce IFN-γ in the Skin during Leishmania Infection. Journal of immunology (Baltimore, Md : 1950). 2018;200:1737-45.

[43] Noben-Trauth N. Susceptibility to Leishmania major infection in the absence of IL-4. Immunol Lett. 2000;75:41-4.

[44] Costa-Silva MF, Gomes LI, Martins-Filho OA, Rodrigues-Silva R, Freire Jde M, Quaresma PF, et al. Gene expression profile of cytokines and chemokines in skin lesions from Brazilian Indians with localized cutaneous leishmaniasis. Mol Immunol. 2014;57:74-85.

Table 1. The results of ANOVA statistical test for ELISA data in the examined groups

Groups	IFN-γ (pg/mL)				IL-4 (pg/mL)				IFN-γ/IL-4 ratio				IgG (µg/mL)			
	Mean ± SD	**95% CI**	**F value**	***p* value**	**Mean ± SD**	**95% CI**	**F value**	***p* value**	**Mean ± SD**	**95% CI**	**F value**	***p* value**	**Mean ± SD**	**95% CI**	**F value**	***p* value**
AB 1[a]	261.9 ± 37.31	236-287	134.4	< 0.0001	184.2 ± 24	168-200.3	19.79	< 0.0001	1.416 ± 0.06	1.37-1.45	469.7	< 0.0001	3068 ± 373	2817-3319	168.3	< 0.0001
AB 2[b]	270.0 ± 42.37	241.5-298.5			175.8 ± 25.7	158.6-193.1			1.505 ± 0.04	1.47-1.53			3778 ± 423	3494-4062		
AB 3[c]	514.0 ± 40.92	486.5-541.5			175 ± 27.3	156.7-193.3			2.94 ± 0.22	2.79-3.1			3984 ± 397	3717-4251		
PC[d]	279.6 ± 39.48	253.1-306.2			244.6 ± 38.8	218.5-270.7			1.14 ± 0.06	1.1-1.18			5626 ± 377	5373-5880		
NC[e]	153.1 ± 27.3	134.8-171.4			135.3 ± 28.5	116.1-154.4			1.13 ± 0.07	1.08-1.18			1522 ± 327	1302-1741		

[a] AB 1: the group that received 1×10^6 apoptotic blebs from *Leishmania major*-infected macrophages

[b] AB 2: the group that received 5×10^6 apoptotic blebs from *Leishmania major*-infected macrophages

[c] AB 3: the group that received 1×10^7 apoptotic blebs from *Leishmania major*-infected macrophages

[d] PC: the immunization control group that received *Leishmania* lysate antigens

[e] NC: the negative control group

Table 2. Pairwise comparison using *post hoc* Tukey's HSD statistical test for ELISA data[a]

IFN-γ (pg/mL)	AB 1	AB 2	AB 3	PC	NC
AB 1[b]	0	8.09	252.1****	17.73	108.8****
AB 2[c]		0	244.0****	9.63	116.9****
AB 3[d]			0	234.4****	360.9****
PC[e]				0	126.5****
NC[f]					0

IL-4 (pg/mL)	AB 1	AB 2	AB 3	PC	NC
AB 1	0	8.36	9.18	60.45***	48.91**
AB 2		0	0.81	68.82****	40.55*
AB 3			0	69.64****	39.73*
PC				0	109.4****
NC					0

IFN-γ/IL-4 ratio	AB 1	AB 2	AB 3	PC	NC
AB 1	0	0.08	1.53****	0.27****	0.28****
AB 2		0	1.44****	0.36****	0.37****
AB 3			0	1.8****	1.81****
PC				0	0.007
NC					0

IgG (µg/mL)	AB 1	AB 2	AB 3	PC	NC
AB 1	0	709.7***	916.2****	2558****	1546****
AB 2		0	206.5	1849****	2256****
AB 3			0	1642****	2463****
PC				0	4105****
NC					0

[a] Mean difference shown

[b] AB 1: the group that received 1×10^6 apoptotic blebs from *Leishmania major*-infected macrophages

[c] AB 2: the group that received 5×10^6 apoptotic blebs from *Leishmania major*-infected macrophages

[d] AB 3: the group that received 1×10^7 apoptotic blebs from *Leishmania major*-infected macrophages

[e] PC: the immunization control group that received *Leishmania* lysate antigens

[f] NC: the negative control group

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

**** $p < 0.0001$

Figure legends:

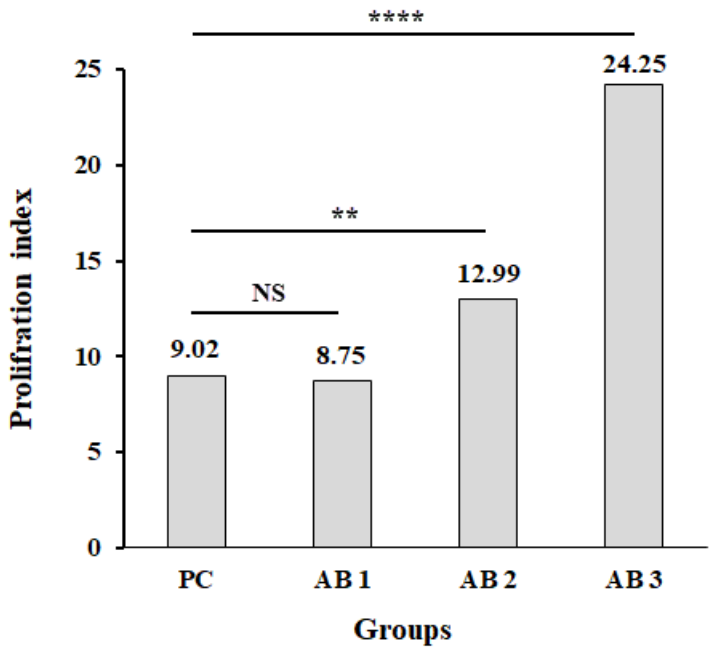
Figure 1. Detection of apoptotic blebs using flow cytometry. Q1: necrotic bodies; Q2: late apoptotic; Q3: live cells; Q4: early apoptotic. The scales represent relative intensity of fluorescence for each of the two fluorophores. The sample was diluted to one-sixtieth

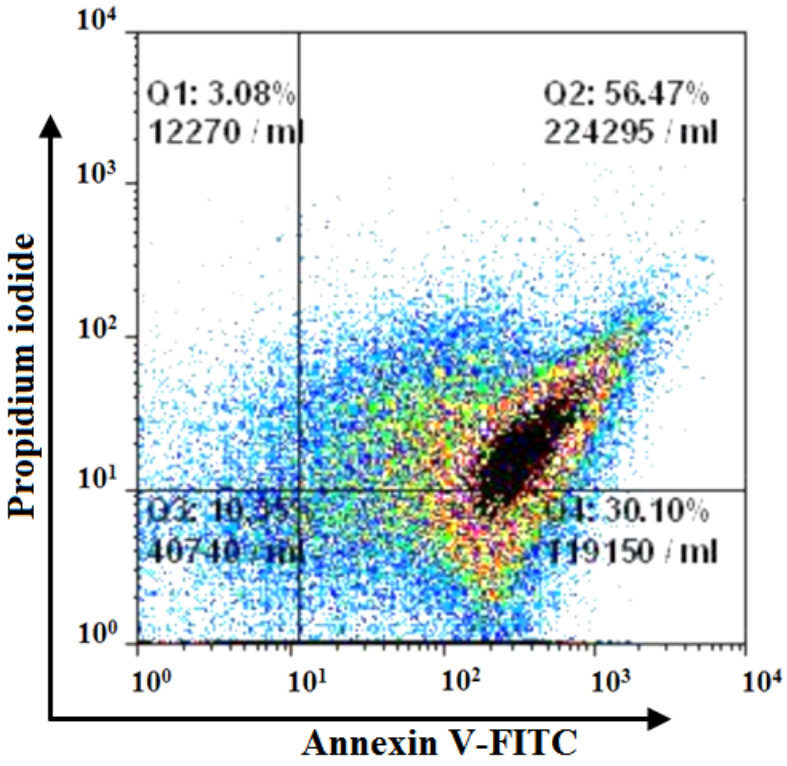
Figure 2. Detection of apoptotic blebs using scanning electron microscope (SEM)

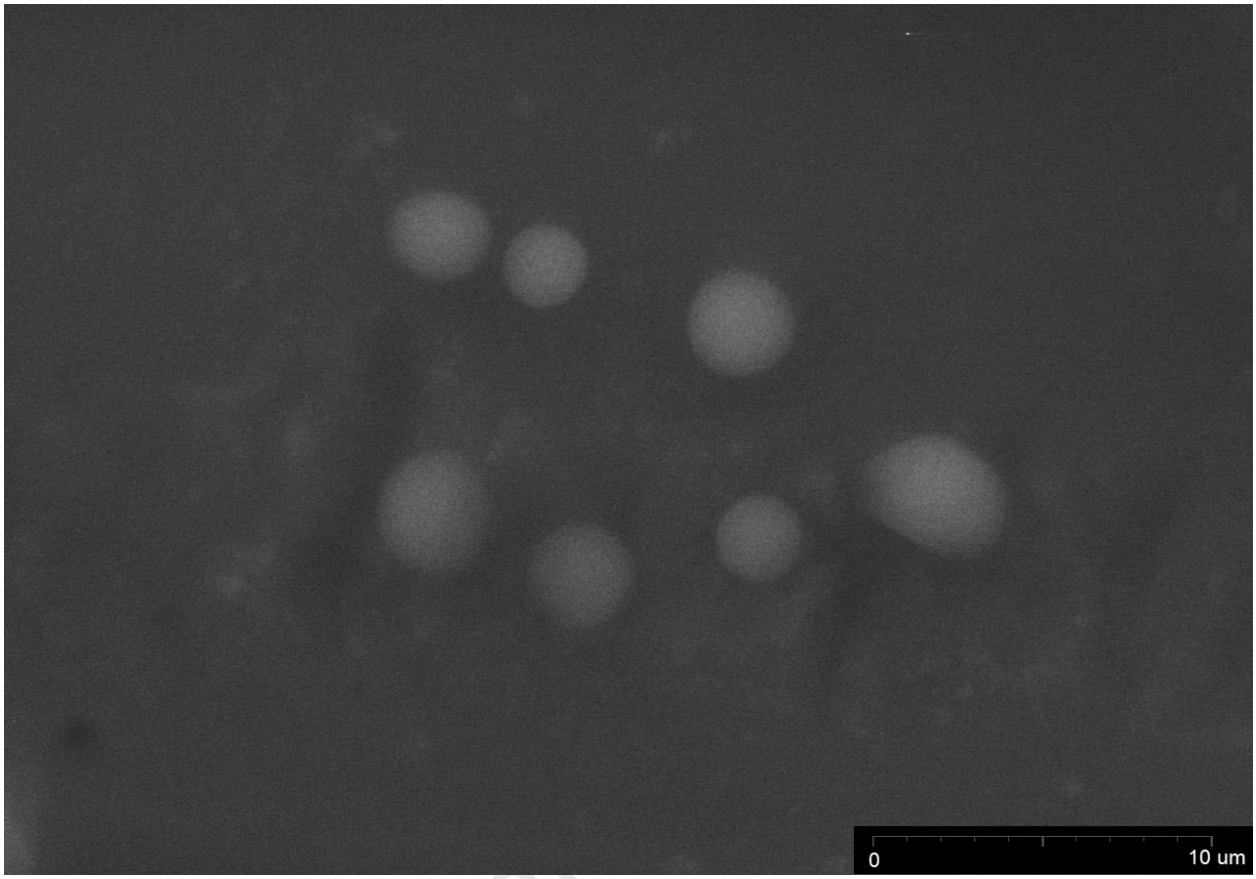
Figure 3. The amount of IFN-γ, IL-4, IFN-γ/IL-4 ratio, and total IgG in the serum samples of the examined mice. AB 1: the group that received 1×10^6 apoptotic blebs from *Leishmania major*-infected macrophages; AB 2: the group that received 5×10^6 apoptotic blebs from *Leishmania major*-infected macrophages; AB 3: the group that received 1×10^7 apoptotic blebs from *Leishmania major*-infected macrophages; PC: the immunization control group that received *Leishmania* lysate antigens; NC: the negative control group

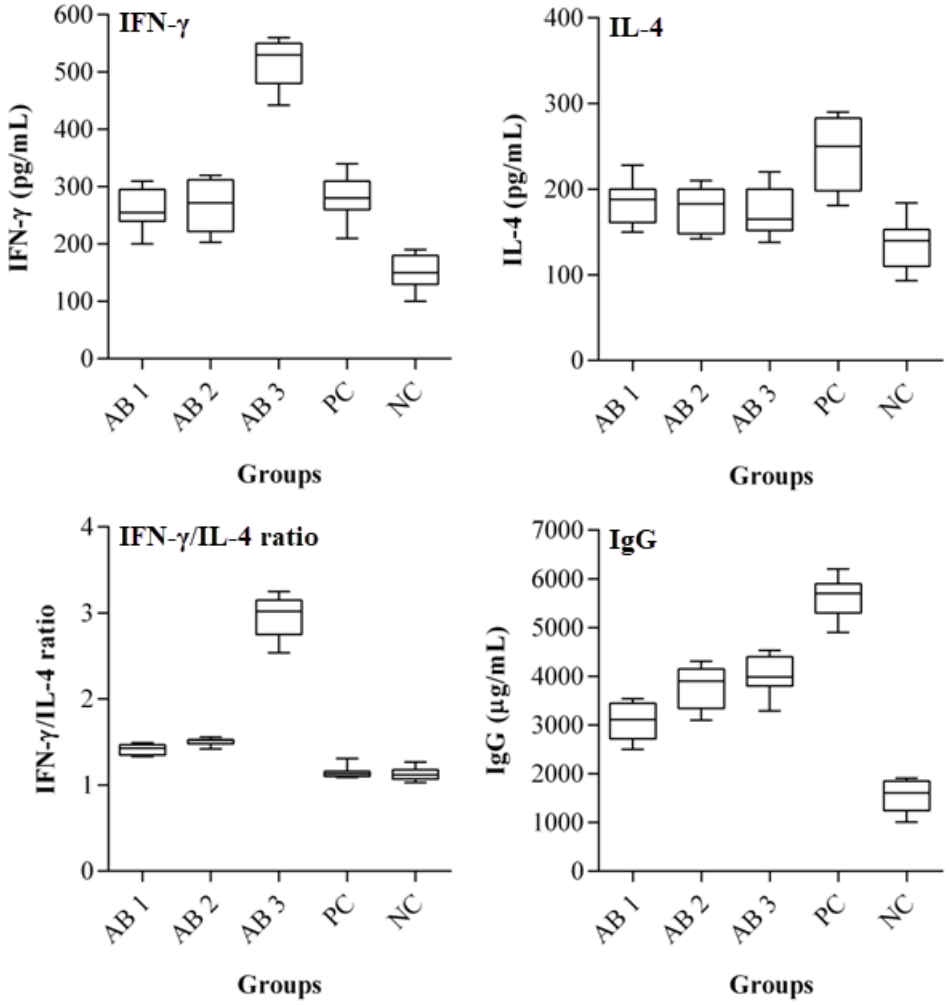
Figure 4. Proliferation index of lymphocytes in the treated groups relative to the negative control group. AB 1: the group that received 1×10^6 apoptotic blebs from *Leishmania major*-infected macrophages; AB 2: the group that received 5×10^6 apoptotic blebs from *Leishmania major*-infected macrophages; AB 3: the group that received 1×10^7 apoptotic blebs from *Leishmania major*-infected macrophages; PC: the immunization control group that received *Leishmania* lysate antigens. ** $p < 0.01$; **** $p < 0.0001$; NS = not significant

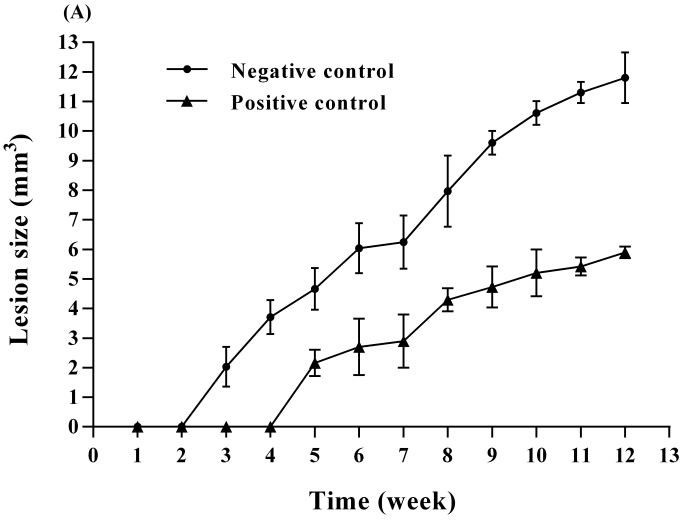
Figure 5. The process of lesion formation from the beginning to the end of the examination. (A) Lesion size in the negative and immunization control groups. (B) Top-left picture shows the lesion in the negative control group. Top-right picture shows the lesion in the immunization control group. The two pictures below show that the mice receiving apoptotic bodies did not develop lesion. Immunization control = LLAs group

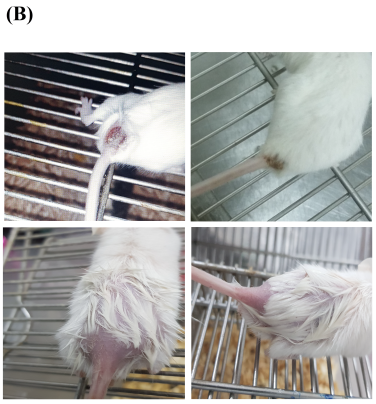







IFN-γ
IFN-γ (pg/mL)
600
500
400
300
200
100
0
AB 1
AB 2
AB 3
PC
NC
Groups
IL-4
IL-4 (pg/mL)
400
300
200
100
0
AB 1
AB 2
AB 3
PC
NC
Groups
IFN-γ/IL-4 ratio
IFN-γ/IL-4 ratio
4
3
2
1
0
AB 1
AB 2
AB 3
PC
NC
Groups
IgG
IgG (µg/mL)
7000
6000
5000
4000
3000
2000
1000
0
AB 1
AB 2
AB 3
PC
NC
Groups





Highlights:

- No lesions were observed in the groups vaccinated with apoptotic blebs from *Leishmania major*-infected macrophages
- Apoptotic blebs contain a wide variety of the parasite antigens and may stimulate immune responses similar to whole killed parasite
- The highest IFN-γ and IFN-γ/IL-4 ratio were observed in the group that received 1×10^7 apoptotic blebs
- The immunization with apoptotic blebs protected mice from cutaneous swelling against *Leishmania major* challenge

Author Statement:

Roghiyeh Faridnia: *Leishmania* Maintenance, Apoptosis Induction, Preparation of *Leishmania* Lysate Antigens, Immunization of Mice. **Hamed Kalani**: Flow Cytometry, Scanning Electron Microscope, Lymphocyte Proliferation Assay, Design of the Study, Drafting the Manuscript. **Hajar Ziaei Hezarjaribi**: Immunization of Mice. **Paul W. Denny**: Writing-Reviewing and Editing. **Alireza Rafie**: Cytokine and Antibody Assay. **Mahdi Fakhar**: Infection Challenge, Data analysis, Writing and Editing. **Stela Virgilio:** Reviewing and Editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: