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Original Article

Fine-Tuning Immune Responses in Cutaneous Leishmaniasis: How *Leishmania major* Antigens Shape Th1/Th2 Differentiation

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Abstract

Introduction: Leishmaniasis, a widespread zoonotic disease caused by various species of the parasitic protozoan *Leishmania*, presents a significant global health challenge. Current treatment options have limitations, prompting research into alternative approaches. Utilizing parasite-derived antigens to stimulate and modulate the host's immune system holds promise as a potential therapeutic strategy. This study investigates the role of *Leishmania major* excretory/secreted antigens (ESA) and lysate *Leishmania* antigens (LLA) in modulating Th1 and Th2 immune responses.

Methods: *Leishmania major* parasites [MRHO/IR/75/ER] were cultured in NNN and RPMI1640 media. LLA and ESA were prepared and quantified for protein content. Subsequently, BALB/c mice were treated with LLA, ESA, and adjuvants (complete and incomplete Freund's adjuvant), either alone or in combination. Cellular immune responses against parasite antigens were then evaluated.

Results: The average weight of mice in the negative control group did not differ significantly from that of mice receiving adjuvant alone ($P>0.05$). However, a significant difference was observed between the negative control group and the groups receiving ESA, LLA, or a combination of both ($P<0.05$).

Conclusion: The findings of this study suggest that ESA and LLA derived from *Leishmania major* play a role in modulating the immune response. The results indicate that the use of these antigens, alone or in combination with adjuvants, can impact mouse weight. While the negative control group did not show a significant difference compared to the adjuvant-only group, significant differences were observed between the negative control group and the groups receiving the antigens. These observations point to the potential of the investigated antigens to stimulate immune responses and could be considered for the development of therapeutic strategies against leishmaniasis. However, further studies are needed to fully elucidate the immunogenic mechanisms and to optimize the use of these antigens.

Keywords: *Leishmania major*, Immune responses, Cutaneous leishmaniasis, Th1/Th2

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Introduction

Leishmaniasis is a tropical infectious disease prevalent in 98 countries, with an estimated 0.7 to 1.3 million annual cases (1). It is caused by over 20 species of *Leishmania* protozoa, presenting primarily in three forms: cutaneous, mucocutaneous, and visceral, with cutaneous leishmaniasis (CL) being the most common (2). Approximately 75% of CL cases are found in ten countries, including Afghanistan and Sudan (3,4). The two dominant types of CL are anthroponotic CL (ACL), caused by *Leishmania tropica*, and zoonotic CL (ZCL), caused by *Leishmania major*. Transmission occurs through infected sandfly bites, and there is currently no vaccine for CL (5). Standard treatment with pentavalent antimonials has significant side effects and a high failure rate (6).

T cells in the lymph nodes that identify and drain the site of infection, due to the presence of IL-12, proliferate and differentiate into Th1 cells (7). These T cells then migrate to the site of the lesion, where they produce IFN-γ, leading to macrophage activation and parasite control. During the early stages of infection, recruited monocytes provide a haven for parasites (8); however, as the infection progresses and immunity develops, infiltrating monocytes are activated by IFN-γ produced by effector T cells and skin-resident memory T cells, contributing to protection (9,10). The importance of IFN-γ and IL-4 cytokines in regulating anti-leishmanial immunity has been extensively studied *in vitro* and *in vivo* (11).

Drug resistance in parasitic diseases, including leishmaniasis, is a primary concern for researchers. Disease management worldwide is too interconnected and complex for efforts to control it effectively. Various factors have contributed to the reduced efficacy of drugs used against leishmaniasis, including changes in associated

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host immunity and shifts in demographics within the disease range.

This study aimed to investigate the role of *Leishmania major* excretory/secreted antigens (ESA) and lysate *Leishmania* antigens (LLA) in modulating Th1 and Th2 immune responses.

Materials and Methods

Cultivation of Parasites and Preparation of Lysate Leishmania Antigen

The *Leishmania major* strain [MRHO/IR/75/ER] was cultivated in NNN medium, followed by mass cultivation in enriched RPMI-1640 medium. For three months, the parasites were continuously cultured in an enriched medium, with a new strain passage every four cycles. This process continued until a population of five billion leptomonads was reached. The cultured parasites were pooled, washed three times in cold PBS with a pH of 7.5, and then incubated in PBS containing 1% Triton X-100. After six cycles of freeze-thawing, they were stored at -70 °C until further processing.

Cultivation of Parasites and Preparation of Excretory-Secretory Antigen

To prepare ESA, *Leishmania major* [MRHO/IR/75/ER] parasites in the stationary phase were collected from fourth-passage single-phase RPMI-1640 medium by centrifugation at 3000 g for 15 minutes. The supernatant was discarded, and the parasites were washed three times with RPMI-1640 medium free of FCS and antibiotics at the same speed and duration. The parasites were transferred to RPMI-1640 without antibiotics or FCS for ESA preparation. ESA sampling was conducted at 0, 6, 12, 24, 48, and 72 hours by centrifuging at 3000 g for 15 minutes. The supernatant was filtered using a 0.22-micron filter to remove any remaining parasites and stored at -70°C until used in assays.

Protein Quantification of ESA and LLA by Bradford Method

The Bradford method, noted for its speed, accuracy, and sensitivity to microgram quantities of protein, was used to quantify protein levels. This method relies on Coomassie Brilliant Blue G-250, which binds specifically to peptide bonds, with maximum absorbance shifting from 465 nm (before binding) to 595 nm (after binding) when read by spectrophotometry.

Assessment of ESA and LLA in BALB/c Mice for Cellular Immune Response Evaluation

Thirty-five female BALB/c mice were randomly divided into five groups of seven. ESA and LLA antigens were prepared such that each mouse received a 100 µl subcutaneous injection in the underarm region along with either Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA). The groups were as follows: First Group: CFA Antigen, Second Group: CFA + ESA Antigen, Third Group: CFA + LLA Antigen, Fourth Group: CFA + ESA & LLA Antigens, Fifth Group: No molecules or adjuvants injected. After two weeks, ESA and LLA were administered with IFA, as follows: First Group: IFA Antigen, Second Group: IFA + ESA Antigen, Third Group: IFA + LLA Antigen, Fourth Group: IFA + ESA & LLA Antigens, Fifth Group: No molecules or adjuvants injected.

Challenge and Evaluation of Lesion Development and Splenomegaly in Mice

Four weeks after the second injection, each mouse received a 0.1 mL subcutaneous injection in the tail containing one million metacyclic promastigotes of Leishmania major (MRHO/IR/75/IR). Five weeks post-infection, nodules began to appear in the first and fifth groups. The wound size and body weight of the mice were recorded weekly from the onset of lesions to track disease progression until complete infection. Spleen width was measured post-mortem for each group.

Results

Evaluation of Excretory-Secretory and Lysate Leishmania Antigens In Vivo

Weight changes across groups: The mean and standard deviation of weight changes were compared across the following groups:

- Negative group: No molecules or adjuvants (IFA & CFA).
- Adjuvant group: Adjuvants only (IFA & CFA).
- ESA group: ESA molecules with adjuvants (IFA & CFA).
- LLA group: LLA molecules with adjuvants (IFA & CFA).
- ESA & LLA group: Both ESA and LLA molecules with adjuvants (IFA & CFA).

ANOVA revealed no significant difference in weight over time among groups 1 to 4 ($P > 0.05$), but a statistically significant difference was observed between group 5 and groups 1–4 ($P < 0.05$). Within-group comparisons also showed a significant difference ($P = 0.001$).

According to the multiple comparisons test and Bonferroni correction, no significant differences were observed in the weights of the Negative group compared to the Adjuvant, ESA, LLA, and ESA + LLA groups during weeks 1 through 4 ($P > 0.05$). However, in week 5, the ESA + LLA group showed a significant difference in weight compared to the ESA, LLA, Adjuvant, and Negative groups ($P < 0.05$). In week 6, the Adjuvant, Negative, ESA, and LLA groups exhibited a significant difference in weight compared to the ESA + LLA group ($P < 0.05$). Still, no significant difference was found within these four groups ($P > 0.05$).

In week 7, the Adjuvant, Negative, ESA, and LLA groups again showed significant differences compared to the ESA + LLA group ($P < 0.05$), but no significant difference

was observed within these groups ($P>0.05$). In week 8, only the Adjuvant group demonstrated a significant difference from the ESA + LLA group ($P<0.05$), while the other groups showed no significant differences among each other ($P>0.05$). From week 11 onwards, all groups—Adjuvant, Negative, ESA, and LLA—showed significant differences in weight compared to each other ($P<0.05$) (Table 1 and Figure 1).

Lesion Diameter Comparison Across Groups

Mean and standard deviation of lesion diameter in mice across groups:

- Negative Group: No molecules or adjuvants .
- Adjuvant Group: Adjuvants only .
- ESA Group: ESA molecules with adjuvants. (Excretory-Secretory Antigen)
- LLA Group: LLA molecules with adjuvants .(Lysate Leishmania Antigen)
- ESA & LLA Group: Both ESA and LLA

ANOVA analysis: Time and mean lesion diameter showed significant differences across all groups ($P=0.0001$). ANOVA and Multivariate Analysis: Time and mean lesion diameter did not show a significant difference within groups 5, 8, and 12, whereas significant differences were observed in the other groups ($P<0.05$).

Multiple Comparisons and Bonferroni Test

During weeks 1 through 4, mean lesion diameter showed a significant difference between Negative, Adjuvant, ESA, and LLA groups compared to the ESA + LLA group

Table 1. Mean and Standard Deviation of Body Weight (in grams) of Mice Receiving Excretory-Secretory and Lysed Antigens of *Leishmania* Parasite over 18 Weeks Post-Infection with Cutaneous Leishmaniasis.

Group					Week
Negative	Adjuvant	ESA	LLA	ESA + LLA	
25.01 ± 0.619	24.98 ± 0.519	24.80 ± 0.437	24.81 ± 0.634	24.67 ± 0.678	1
24.65 ± 0.610	24.61 ± 0.605	24.46 ± 0.628	24.74 ± 0.636	24.67 ± 0.648	2
24.34 ± 0.683	24.45 ± 0.665	24.32 ± 0.632	24.54 ± 0.609	24.86 ± 0.886	3
24.26 ± 0.655	24.32 ± 0.668	24.21 ± 0.640	24.53 ± 0.642	24.98 ± 0.879	4
23.98 ± 0.586	24.20 ± 0.648	24.09 ± 0.630	24.39 ± 0.627	25.14 ± 0.846	5
23.83 ± 0.585	23.99 ± 0.610	23.85 ± 0.651	24.26 ± 0.626	25.32 ± 0.868	6
23.49 ± 0.568	23.75 ± 0.597	23.69 ± 0.648	24.10 ± 0.626	25.56 ± 0.876	7
23.21 ± 0.596	21.66 ± 6.722	23.50 ± 0.666	23.93 ± 0.637	25.82 ± 0.887	8
23.10 ± 0.592	23.44 ± 0.567	23.34 ± 0.632	23.73 ± 0.624	26.19 ± 0.953	9
22.69 ± 0.553	23.24 ± 0.545	23.16 ± 0.641	23.61 ± 0.530	26.47 ± 0.969	10
22.43 ± 0.586	23.03 ± 0.524	22.98 ± 0.643	23.37 ± 0.538	26.78 ± 0.981	11
22.11 ± 0.591	22.71 ± 0.524	22.81 ± 0.630	23.12 ± 0.626	27.06 ± 1.000	12
21.73 ± 0.775	22.21 ± 0.565	22.58 ± 0.585	22.87 ± 0.647	27.43 ± 0.985	13
21.13 ± 0.776	21.66 ± 0.850	22.19 ± 0.489	22.58 ± 0.684	27.80 ± 1.026	14
20.05 ± 0.725	20.31 ± 0.699	21.79 ± 0.351	22.17 ± 0.736	28.41 ± 1.930	15
.00 ± .000	.00 ± .000	21.40 ± 0.333	21.60 ± 0.880	28.77 ± 1.057	16
00 ± .000.	.00 ± .000	.00 ± .000	.00 ± .000	29.43 ± 1.117	17
00 ± .000.	.00 ± .000	.00 ± .000	.00 ± .000	30.44 ± 1.201	18

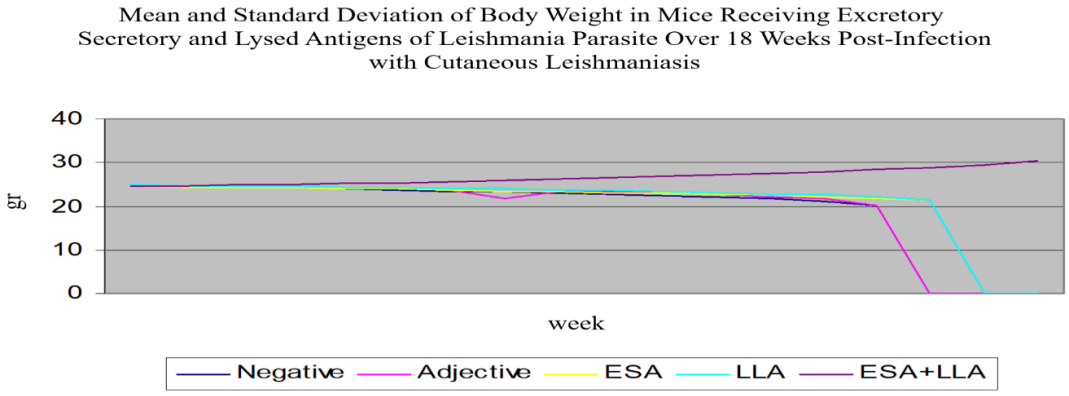

Figure 1. Mean and Standard Deviation of Body Weight (in grams) of Mice Receiving Excretory-Secretory and Lysed Antigens of *Leishmania* Parasite over 18 Weeks Post-Infection with Cutaneous *Leishmaniasis*

($P<0.05$), with no significant differences among the other groups ($P>0.05$). From week 5 to week 18, mean lesion diameter showed a significant difference across all five groups (Negative, Adjuvant, ESA, LLA, and ESA+LLA) ($P<0.05$) (Table 2 and Figure 2).

Spleen Width Comparison Across Groups

The average spleen width in the negative mice group was 4.39±0.12 mm, in the adjuvant mice group was 4.41±0.07 mm, in the mice group receiving excretory-secretory antigen was 4.49±0.27 mm, in the mice group receiving lysate antigen was 4.44±0.17 mm, and in the mice group receiving excretory-secretory and lysate antigens was 4.53±0.11 mm. No significant difference was observed in terms of statistical analysis ($P<0.05$) (Table 3 and Figure 3).

Discussion

The positive effects of parasites and their derivatives have garnered attention in recent studies, with excretory substances secreted by parasites showing promise in immune regulation, anti-cancer properties, and wound healing (12). In the present study, the effects of excretory-secretory antigens and *L. major* parasite lysate on wound healing and weight gain in laboratory animal models were evaluated (13).

Our findings indicated a significant difference in the average weight of mice treated with excretory-secretory, lysate, and combined antigens ($P<0.05$). Additionally, the average wound diameter in the negative control group

Table 2. Mean and Standard Deviation of Lesion Diameter (in millimeters) in Mice Receiving Excretory-Secretory and Lysed Antigens of Leishmania Parasite Over 18 Weeks Post-Infection with Cutaneous Leishmaniasis

Group					Week
Negative	Adjuvant	ESA	LLA	ESA+LLA	
0.018±.1940	0.015±.1910	49.50±0.406	0.333±.3220	0.183±.6800	1
0.120±.2620	0.017±.2120	0.396±.4160	0.266±.2460	0.187±.6800	2
0.057±.2520	0.016±.2450	0.014±.1230	0.241±.2170	0.251±.6310	3
0.060±.3000	0.021±.2810	0.015±.1500	0.027±.1640	0.360±.6030	4
10.954±3.824	0.024±.3250	0.017±.1790	0.027±.1990	0.292±.2520	5
0.066±.4130	0.027±.3700	0.023±.2110	0.037±.2320	0.181±.1860	6
0.066±.4740	0.036±.4140	0.025±.2480	0.034±.2660	0.237±.2260	7
15.324±5.385	0.040±.4570	0.026±.2840	0.032±.3020	0.021±.1600	8
0.072±.6010	0.046±.5140	0.032±.3210	0.035±.3460	0.027±.1790	9
0.079±.6690	0.051±.5610	0.031±.3630	0.038±.3800	0.026±.2010	10
0.071±.7190	0.044±.6080	0.043±.4040	0.042±.4230	0.026±.2240	11
923.468±8.20	0.041±.6630	0.043±.4470	0.037±.4610	0.055±.2620	12
0.069±.843	0.035±.720	0.040±.496	0.040±.505	0.051±.274	13
0.075±.8950	0.034±.7670	0.042±.536	0.034±.550	0.053±.2930	14
0.280±.8725	0.054±.8060	0.051±.573	0.042±.6000	0.046±.3190	15
.000±.00	.047±.890	.052±.620	0.047±.65	0.040±.34	16
.000±.00	.000±.00	.000±.00	0.000±.00	0.041±.38	17
.000±.00	.000±.00	.000±.00	0.000±.00	0.048±.42	18

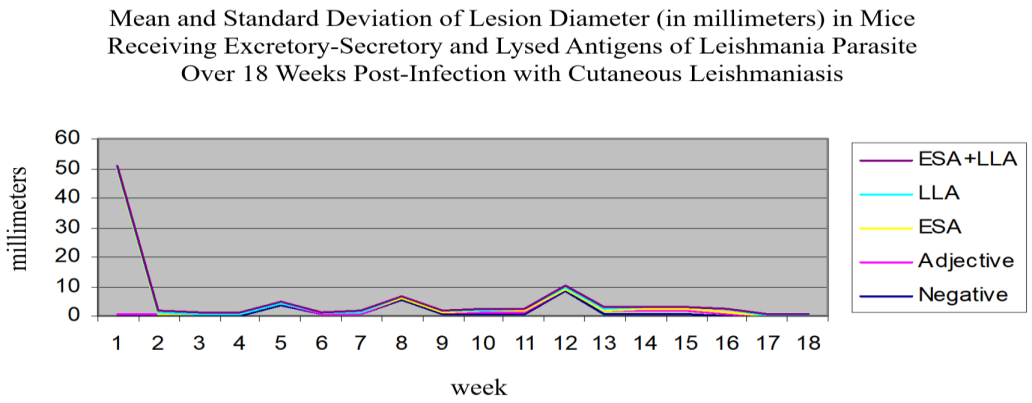

Figure 2. Mean and Standard Deviation of Lesion Diameter (in millimeters) in Mice Receiving Excretory-Secretory and Lysed Antigens of Leishmania Parasite over 18 Weeks Post-Infection with Cutaneous Leishmaniasis

Table 3. Mean Spleen Width (in millimeters) in Mice Receiving Excretory-Secretory and Lysed Antigens of Leishmania Parasite Over 18 Weeks Post-Infection with Cutaneous Leishmaniasis. *P*-value 0.305.

The Width of the Spleen	The Experimental Group of Mice
0.12 ± 4.39	Negative
0.07 ± 4.41	Adjuvant
0.27 ± 4.49	ESA
0.17 ± 4.44	LLA
0.11 ± 4.53	ESA + LLA

showed a significant difference ($P < 0.05$) compared to the group receiving excretory-secretory and lysate antigens. Conversely, the average wound diameter in the adjuvant group and the groups receiving excretory-secretory or lysate antigens alone did not show significant differences ($P > 0.05$).

Similarly, Kovner et al. highlighted the potential of excretory-secretory derivatives and lysates of the foodborne trematode *Opisthorchis felineus* (14). Their study demonstrated that after 10 days of *in vivo* testing/challenge, the percentage of wound healing in certain treatment groups significantly exceeded the control values. Furthermore, wound treatment with excretory-secretory products and worm lysate resulted in (*a*) reduction of inflammation, (*b*) modulation of vascular response, and type 1 collagen deposition promoting dermal ECM remodeling. Infection with some parasites that have secretions, such as growth factors, can act as a double-edged sword. On one hand, it promotes wound healing and tissue repair; on the other hand, it can lead to carcinogenic effects and uncontrolled hyperplasia/neoplasia. According to the study of Giraud et al., the proteophosphoglycans gel plaque derived from *Leishmania* in the sand fly showed promising results in accelerating dermal wound healing *in vivo* (15).

Based on these interpretations, compounds derived from certain parasites may offer potential for tissue repair (non-malignant) through optimized application. However, in the present study, the raw lysate of *L. major*, the parasite itself causing tissue complications, with an amalgam of protein compounds and other substances, significantly aided wound healing (16). Experimental trials are conducted to evaluate the effect and interaction of these compounds with wound microenvironments or even malignant tissues through mediators and/or immune responses. Nowadays, it is common to use compounds of parasitic origin in crude form (such as lysate) (17); in this regard, the application of excretory-secretory antigens against different cancer cell lines was investigated. This study concluded that by adjusting the concentration of parasite derivatives and optimizing with novel approaches (such as combining with nanoparticles), the effects of parasitic compounds can be enhanced.

To apply the synergistic effect, multiple error tests are proposed, and in the present research, the impact of lysates

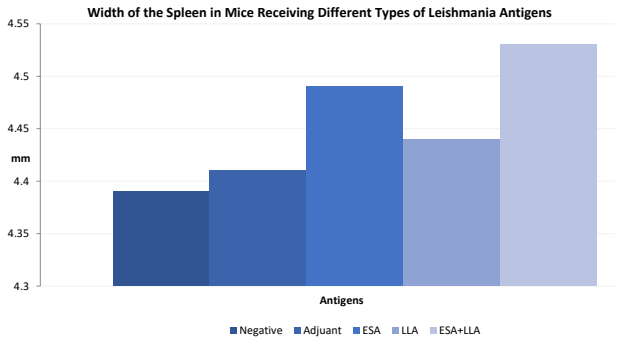

Figure 3. Mean Spleen Width (in millimeters) in Mice Receiving Excretory-Secretory and Lysed Antigens of Leishmania Parasite over 18 Weeks Post-Infection with Cutaneous Leishmaniasis

and ES antigens was investigated alone and together. The latter strengthened the effect, which, according to similar previous studies, was expected. Ribro et al. investigated the impact of *Neospora caninum* ES antigens and lysate alone, combined with CpG oligodeoxynucleotides (ODN) (as an immunoadjuvant). However, contrary to our findings, they found that CpG-ODN combined with *N. caninum* lysate, but not with ES antigen, enhances protection against infection in mice (18-22). It seems that more and more comprehensive studies are needed to confirm the present findings and draw conclusions about the effect of parasite derivatives.

Conclusion

Despite the promising outcomes of *Leishmania*-origin lysate and ES antigens on wound healing and weight gain, further investigations should be conducted at the cellular and even molecular levels. A deeper understanding of the immunogenic and protective components, as well as their interactions with the immune system, will lead to more robust conclusions. Additionally, antigens utilized in future studies should consist of a combination of adjuvants, immunogens, and macrophage proliferators to stimulate the immune system effectively against the Leishmania parasite.

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Validation: Mojtaba Norouzi.

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Competing Interests
The authors declared no competing interests.

Ethical Approval
Ethical approval to perform this study was obtained by the Ethical Review Board of Yazd Shahid Sadoughi University of Medical Sciences (Ethic Code: IR.SSU.MEDICINE.1395.169).

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