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**RESEARCH ARTICLE**

# Immunoassay of IL-10 and IL-2 in mice model vaccinated with killed *Leishmania major* vaccine.

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## *Abstract*

Cutaneous leishmaniasis is caused by protozoan parasites of the genus Leishmania,*L.major* (wet cutaneous leishmaniasis) and *L.tropica* (dry cutaneous leishmaniasis).They are transmitted as promastigotes to mammalian hosts by the bite of infected female phlebotomine sand flies**.**The inoculation of mice with *L.major* experimentally reproduces cutaneous leishmaniasis and the development of a polarized Th1 or Th2 response results in resistance or susceptibility,respectively to this intracellular parasite.Vaccines against the *L.major* have historically progressed from the use of living virulent organisms to attenuated and then to killed promastigotes.A total of 45 female BALB/c mice at 8-9 weeks of age were grouped as following,first group act as,control uninfected group, second group were infected with 0.1 ml of RPMI 1640 media (each ml of media has $10^7$ promastigote)per mouse,while the third group was Injected with prepared leishmania vaccine.IL-2 and IL-10 titers in mice serum were measured by using of ELISA technique.High levels of IL-0 cytokine were found in the vaccinated mice groups, these ranged from (3.36 to 4.99 pg/ml) in the first week after vaccination,(9.57 to 10.95 pg/ml)after $10^{th}$ day of challenge infection,and in the $20^{th}$ day they ranged from (11.01 to 12.03 pg/ml),while in the control and infected groups they ranged from (0.99 to 2.02 pg/ml) and (3.02 to 4.89 pg/ml) respectively.Titer of IL-2 cytokine of the vaccinated mice groups, after challenge infection was ranged from (3.10 to 3.58 pg/ml) in the first week, and from (3.59 to 3.69) after the$10^{th}$ day which was in the same range of the infected mice group(3.01-3.58 pg/ml).while in the $20^{th}$ day after challenge infection it ranged from (3.67 to 4.12 pg/ml).

**Conclusion:** we concluded that prepared killed vaccine, increases production of IL-10 (Th2 immune response) in vaccinated mice group, in comparison with infected group which provides protection in mice against virulent *L. major* challenge.There is no lesions of *L.major* infection appear on mice which challenged with *L.major* after vaccination

## Introduction

Leishmania are intracellular parasites that reside primarily within host tissue macrophages **(Green et al., 1990).** There are two developmental forms of Leishmania:the promastigote and the amastigote **(Kane and Mosser, 2001).** The promastigote is introduced into the mammalian host when an infected female sand fly takes a blood meal. This form is taken up by phagocytic cells and rapidly transforms into the amastigote form.Amastigotes replicate intracellularly within mononuclear phagocytes and are the only form found within the mammalian host following infection. (CL) is the most common form of leishmaniasis and causes ulcers on exposed parts of the body, leaving life-long scars and serious disability **(WHO, 2014)**.About 95% of Cutaneous Leishmaniasis (CL) cases occur in the Americas, the Mediterranean basin, and the Middle East and Central Asia. Over two-third of CL new cases occur in six countries: Afghanistan, Algeria, Brazil, Colombia, Iran and the Syrian Arab Republic. An estimated 0.7 million to 1.3 million new cases occur worldwide annually. **(WHO,2014**). The fact that recovery from infection confers immunity to reinfection suggests that control of leishmaniasis by vaccination may be possible **(Handman, 1999).**There are no vaccines in routine use **(Chevalier et al., 2000)**. To date, several approaches to anti-leishmanial vaccine have been tested. First generation vaccines composed of whole killed parasites have been proposed. However, most of the vaccine studies concentrate on the second generation vaccines consisting of recombinant proteins, poly-proteins or dendritic cells loaded with peptides derived from leishmanial antigens. Third generation vaccines are DNA vaccines coding for a specific protein gene cloned in to a vector and injected directly in to the host with continued production of the relevant protein **(WHO, 2010).**Studies of *L. major* infection in inbred mice underpin the Th1/ Th2 paradigm was conducted. Primary immunity to *L. major* in resistant mice requires the induction of a polarized Th1 response **(Kaye et al., 1991 and Louzir et al., 1998).** Recent studies demonstrate that IL-10 is the important regulatory cytokine involved in parasite persistence in mice **(Noazin et al., 2008)**, the major source of which is CD4_CD25_ regulatory T cells (Tr) 3 **(Ahuja et al.,1999)**. Indeed, several studies now demonstrate a role for Tr in modulating both Th1 and Th2 **(Peters and Sacks, 2009)** activity in murine *L. major* infection. This important regulatory role of IL-10 and Tr led us to re-evaluate the key T cells and cytokines that are associated with vaccine failure or success in the susceptible BALB/c *L. major* infection model, IL-10 levels relative to IFN-γ provide the best pre challenge predictor of vaccine success in this model **(Peters et al., 2008).** IL-10 is a multifunctional cytokine produced by a variety of hematopoietic cells, including Macrophages. It has been identified as an immunosuppressive factor that can cause inhibition of antimicrobial activities in mouse and human Macrophage **(Gautam,2010)**.Administration of anti-IL-2 or anti-IL-2 receptor antibody ameliorate the *L.major* infection indicating that IL-2 may also be a susceptibility factor for leishmaniasis **(Henizel et al., 1993).**

## Materials and Methods

### Laboratory animals

Fourty five female mice aged from 8 to 16 weeks old were challenged with *Leishmania major.*

### Leishmania parasite

*Leishmania major* strain (Strain Zymowme LON4) was used for the vaccine challenge.
Experiments and preparation of freeze-thawed Leishmania antigen.*Leishmania major* was cultured in RPMI1640 medium at 26 $^{o}$C supplemented with fetal bovine serum (FBS) (Sera Laboratories International, Horsted Keynes, UK) 100U /mL penicillin + 100 mg/mL streptomycin (BioWhittaker, Verviers, Belgium) and 1% L-glutamine in 25 ml culture flasks.

### Preparation of Leishmania antigen .

Leishmania antigen was obtained from logarithmic phase cultures of promastigotes and these parasites were washed twice in PBS. The pellets obtained were submitted to seven cycles of freezing in liquid nitrogen followed by thawing at 37°C.The preparation was observed under microscope for the presence of intact parasites. Protein content of preparations was determined by the **(Lowry method,1951)** and adjusted to 1 mg/ml protein. Antigen preparation was stored frozen at 70°C and thawed immediately before use.

### Experimental protocol.

A total of 45 BALB/c mice were grouped as following :
**1-Group 1:** Control uninfected group (15 mice).
**2-Group 2:**Infected group (15 mice).Mice were infected at 8-9 weeks of age by intramuscular (IM) injection of 0.1 ml of RPMI 1640 media (each 1ml of media has $10^7$ promastigote) per mouse. Mice developed a lesion that first became clinically detectable around 3-4 weeks post infection. The growth of the lesion was assessed.

**3-Group 3:** Injected with prepared Leishmania vaccine (15 mice).
Mice were vaccinated by subcutaneous route with three doses of the vaccines (with a one week interval), based on (**Santos et al., 2003)** and (**Palatnik et al., 2009**).
Mice from saline solution groups (control and infected) were inoculated with 200 µL of PBS.

**Challenge infection:**
Third group of mice that were previously exposed to prepared vaccine of L. major parasites (3 times one week interval) were challenged one week after the third exposure of vaccine with $10^7$ promastigotes given subcutaneous into the left hind footpad .The next day, recipient mice were challenged with $10^7$ promastigotes **(Liew et al., 1982).**

**Enzyme-linked immunosorbent assay (ELISA) for cytokines:**

**Quantification of IL-10 and IL-2 serum level:**

**Serum collection:**

Serum was collected and Cytokines level were determined by sandwich enzyme-linked immunosorbant assays according to the manufacturer's instructions.Kits of (IL-2 & IL-10) that were obtained from (R&D systems,Inc .Minneapolis,MIN USA).

**Time of serum collection:**
Serum was collected after 4 weeks in group 1 and group 2.In group 3 we collected serum three times as following:
-1 week after the $^{3rd}$ exposure to vaccine (at this day we start the challenge infection).
-10 days post the challenge infection.
-20 days post the challenge infection.

## Results

**Detection to IL-10 levels in mice serum samples**

IL-10 serum level of infected mice was higher than control mice group. IL-10 titer of infected mice was ranged from (3.02 to 4.89 pg/ml), while in control group was ranged from (0.99 to 2.02 pg /ml) as shown in table (1).

Table (1)**. IL-10 level in mice serum samples of group 1(control group) and group 2 (infected group) 4 weeks after infection.**

| Sample no. | IL-10 (pg/ml) titer group 1 | IL-10 (pg/ml) titer group 2 |
|---|---|---|
| **1** | 1.79 | 3.69 |
| **2** | 1.05 | 3.02 |
| **3** | 1.19 | 3.45 |
| **4** | 1.77 | 4.05 |
| **5** | 1.66 | 4.34 |
| **6** | 0.99 | 3.99 |
| **7** | 1.83 | 3.89 |
| **8** | 1.89 | 3.50 |
| **9** | 1.74 | 4.35 |
| **10** | 1.56 | 3.67 |
| **11** | 1.33 | 4.89 |
| **12** | 1.90 | 4.73 |
| **13** | 1.67 | 3.88 |
| **14** | 1.89 | 3.25 |
| **15** | 2.01 | 4.39 |

High levels of IL-10 cytokine were found in all the vaccinated mice groups, these ranged from (3.36 to 4.99 pg/ml) in the first week after vaccination, and IL-10 serum level was increased after $10^{th}$ day of challenge infection with a range from (9.57 to 10.95 pg/ml),while in the $20^{th}$ day after challenge infection ,the increase was ranged from (11.01 to 11.97 pg/ml), as shown in table (2) .

**Table (2) . IL-10 titer in mice serum samples of group 3 (vaccinated group).**

| Sample no. | IL-10 titer group(3) 1 week after the 3rd exposure to vaccine | IL-10 titer group (3) 10 days post the challenge infection | IL-10 titer group (3) 20 days post the challenge infection |
|---|---|---|---|
| **1** | 3.70 | 10.73 | 11.20 |
| **2** | 3.36 | 9.57 | 11.06 |
| **3** | 3.49 | 10.45 | 11.79 |
| **4** | 4.60 | 10.05 | 11.01 |
| **5** | 4.42 | 10.34 | 11.13 |
| **6** | 4.04 | 10.39 | 11.41 |
| **7** | 4.16 | 10.89 | 11.67 |
| **8** | 4.01 | 10.50 | 12.03 |
| **9** | 4.99 | 10.95 | 11.97 |
| **10** | 3.86 | 10.67 | 11.23 |
| **11** | 4.91 | 10.89 | 11.70 |
| **12** | 4.90 | 10.73 | 11.41 |
| **13** | 4.17 | 10.88 | 11.39 |
| **14** | 3.89 | 10.85 | 11.78 |
| **15** | 4.91 | 10.79 | 11.94 |

The obtained data revealed notably higher levels of IL-10 were observed in the vaccinated animal group as compared to the control and infected groups as shown in figure (1).

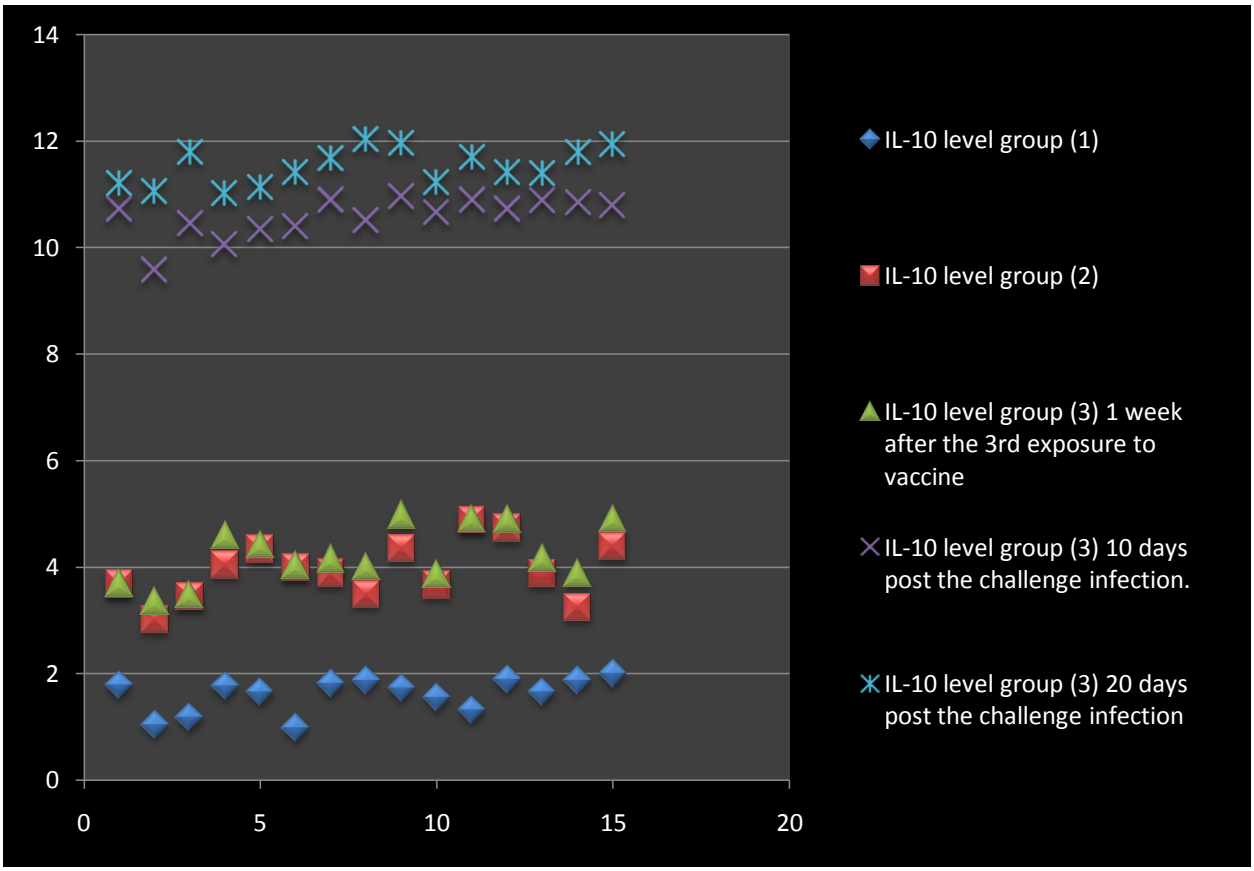


**Figure (1):** Showing serum level of IL-10 in different mice groups.

**Detection to IL-2 serum level:**

The obtained data from this study revealed an increase in serum level of IL-2 of infected mice group in comparison to control mice group. IL-2 titer of infected mice was ranged from (3.01 to 3.58 pg/ml), while ranged from (1.91 to 2.87 pg/ml) in control mice group,as shown in table (3).

**Table (3). IL-2 titer in mice serum samples of group 1(control group) and group 2 (infected group).**

| Sample no. | IL-2 (pg/ml) titer group 1 | IL-2 (pg/ml) titer Group 2 |
|---|---|---|
| **1** | 2.72 | 3.58 |
| **2** | 2.59 | 3.50 |
| **3** | 2.19 | 3.42 |
| **4** | 2.07 | 3.01 |
| **5** | 2.23 | 3.37 |
| **6** | 2.87 | 3.19 |
| **7** | 1.98 | 3.30 |
| **8** | 2.67 | 3.28 |
| **9** | 2.49 | 3.25 |
| **10** | 2.26 | 3.10 |
| **11** | 2.34 | 3.39 |
| **12** | 1.91 | 3.31 |
| **13** | 2.67 | 3.19 |
| **14** | 2.80 | 3.23 |
| **15** | 2.63 | 3.37 |

Titer of IL-2 cytokine of all the vaccinated mice groups, was in the same range of the infected group, this ranged from (3.10 to 3.58 pg / ml) in the first week after vaccination and after $10^{th}$ day of challenge infection was ranged from (3.59 to 3.69 pg/ml), while in the $20^{th}$ day after challenge infection there was an increase where it was ranged from (3.67 to 4.12 pg/ml), as shown in table (4) and figure (2).

**Table (4). IL-2 titer in mice serum samples of group 3 (vaccinated group).**

| Sample no. | IL-2 titer group (3) 1 week after the 3rd exposure to vaccine | IL-2 titer group (3) 10 days post the challenge infection | IL-2 titer group (3) 20 days post the challenge infection |
|---|---|---|---|
| **1** | 3.58 | 3.66 | 4.12 |
| **2** | 3.50 | 3.65 | 4.09 |
| **3** | 3.11 | 3.69 | 3.69 |
| **4** | 3.37 | 3.59 | 3.67 |
| **5** | 3.19 | 3.60 | 3.68 |
| **6** | 3.30 | 3.61 | 3.69 |
| **7** | 3.28 | 3.64 | 3.68 |
| **8** | 3.25 | 3.60 | 3.67 |
| **9** | 3.10 | 3.62 | 3.68 |
| **10** | 3.39 | 3.60 | 3.67 |
| **11** | 3.31 | 3.62 | 3.69 |
| **12** | 3.19 | 3.64 | 3.71 |
| **13** | 3.23 | 3.65 | 3.77 |
| **14** | 3.37 | 3.62 | 3.68 |
| **15** | 3.38 | 3.63 | 3.70 |

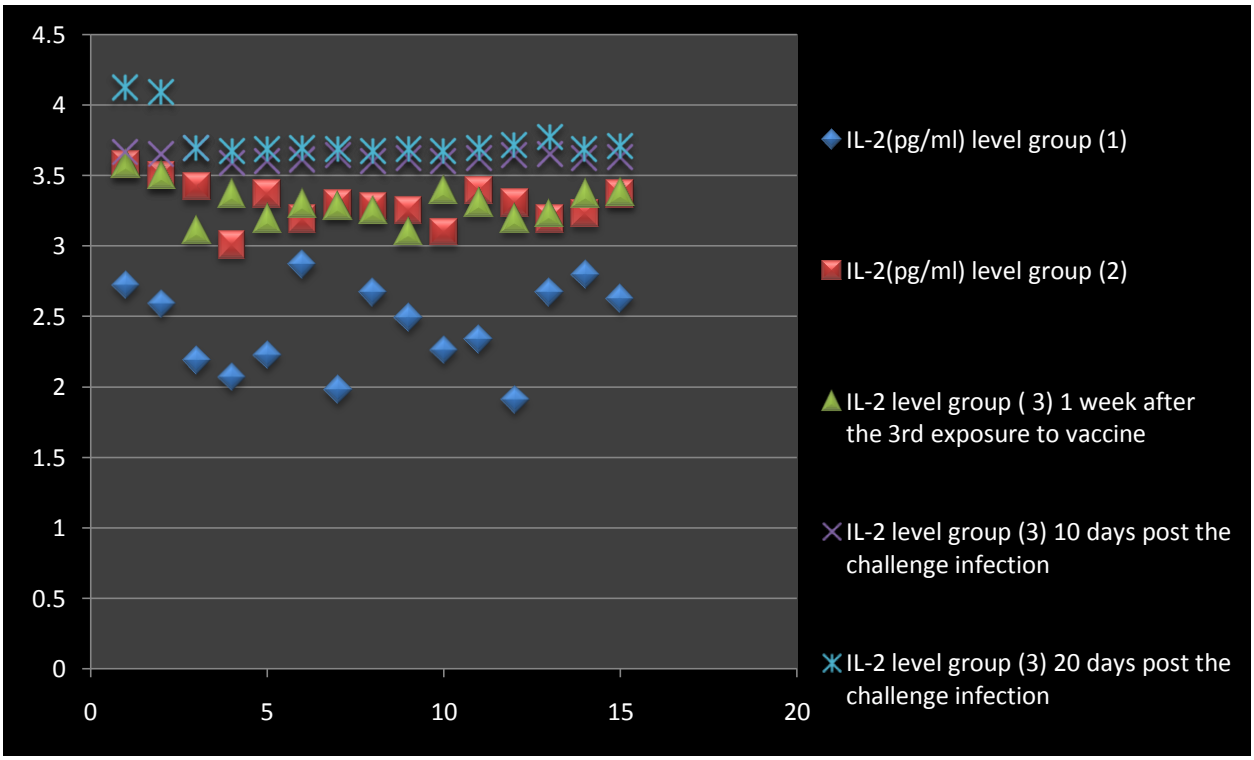


**Figure (2):** Showing serum level of IL-2 in different mice groups.

In comparison between serum level of IL-10 and IL-2 at 20 days post challenge infection, there was considerable increase in IL-10 level than serum level of IL-2, as shown in figure (3).

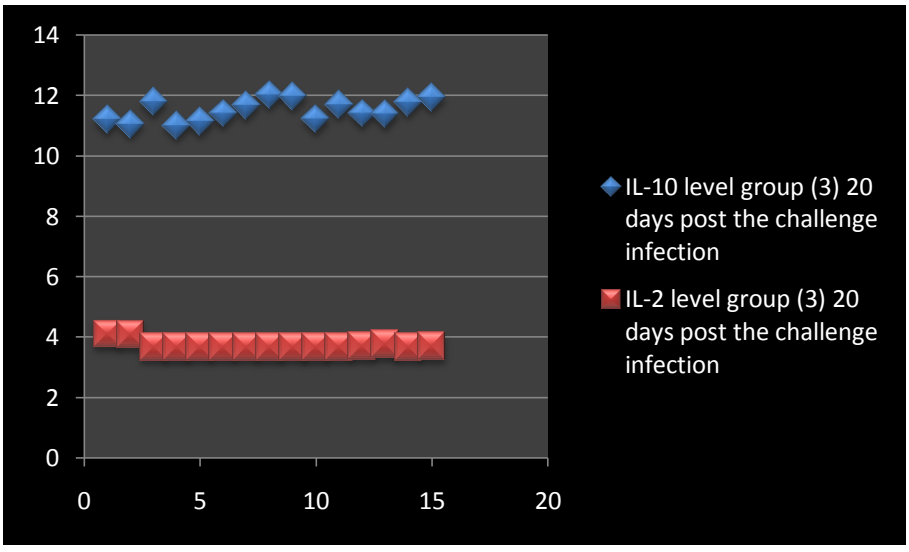


**Figure (3):** Showing increase serum level of IL-10 in comparison to IL-2 after 20 days of challenge infection.

**Challenge infection:**

The obtained data revealed that, there is no signs of *L.major* infection appear on mice under challenge infection with *L.major* 1 week after vaccination. Data indicated that, prepared killed *L.major* vaccine, increase production of IL-10 (Th2 immune response) that provides protection against *L.major* infection.

## Discussion:

Results presented here demonstrate the success of killed *L.major* vaccine to protect against *L.major* (Strain Zymowme LON4) challenge following vaccination, results presented here demonstrate that, IL-10 serum level of infected mice was significant high in comparison to control mice group and IL-10 titer of infected mice was ranged from (3.02 to 4.89 pg/ml),these result was agreed with (**Sutterwala et al.,1998** who mentioned that, IL-10 has important regulatory role during *L.major* infection,IL-10 high titer provide the best pre challenge predictor of vaccine success in murine model.

The findings described here, go with those reported by (**Belkaid et al., 2001; Kane and Mosser., 2001, Murphy et al.,2001**) and previous results with other diverse pathogens **(Moore et al.,2001),** together re-emphasize the breadth of IL-10's regulatory effects in experimental infections. IL-10's actions appear particularly relevant in promoting intracellular infections in which host defense is governed by Th1 cells, mediated by cytokines including IL-12and IFN-γ and requires activated macrophages. In such infections, reducing IL-10 activity almost invariably increases resistance **(Moore et al., 2001),**even in the host with an established,apparently satisfactory Th1 cell response.**(Carmel et al., 2005)** found that absolute levels of IL-10 did predict vaccine success or failure and in particular that the ratio of IFN-γ:IL-10 provide a good pre challenge comparative indicator of vaccine success in the mice model.

T regulatory population follows on from the recent studies demonstrating the importance of T regulatory population **(Belkaid et al., 2002 and Belkaid,2003),**and IL-10 **(Belkaid et al.,2001)** in particular, in determining parasite persistence and immunity to infection.

The obtained results revealed that, IL-2 cytokine of all the vaccinated mice groups, was ranged from (3.11 to 3.58 pg /ml) in the first week after vaccination, and after the10th day of challenge infection was ranged from (3.60 to 3.66 pg/ml), while in the 20th day after challenge infection the increase was ranged from (3.67 to 4.12 pg/ml) .These results was agreed with (**Henizel et al., 1993).**

Data were obtained from these study revealed presence of resistance to *L.major* challenge infection after vaccination, these result was agreed with **(Fred, 1997)** who mentioned that using prepared vaccines against the *L.major* in animal model elicited substantial resistance to subsequent challenge with virulent *L.major* without the inconvenience of long-term persistence of infective organisms.

Our studies have revealed that, prepared killed *L.major* vaccine, increased production of IL-10 (Th2 immune response) in vaccinated mice group, in comparison with infected group.

## Conclusion:

Overall, our results show that vaccination with *L. major* killed vaccine provides protection in mice against virulent *L.major* challenge. Although additional work needs to be done with these vaccine candidates, they offer hope for the development of a killed vaccine against *L.major*.

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