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# Evaluation of the expression of the inflammasome pathway related components in Leishmania major-infected murine macrophages

**M. Mahmoudian Sani[1], A. Hajizade[2], M. Sankian[1], A. Fata[1], M. Mellat[3], K. Hassanpour[4] and Gh. Farnoosh[3]***

[1]*Mashhad University of Medical Sciences, Iran*
[2]*Imam Hossein University, Iran*
[3]*Baquyatallah University of Medical Sciences, Iran*
[4]*Sabzevar University of Medical Sciences, Iran*

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**ABSTRACT**

*Leishmania major is an intracellular parasite that is transmitted to humans by the bite of female phlebotomine sandflies. It causes cutaneous leishmaniasis and has the ability of surviving in macrophages. It has been shown that NOD-like Receptors (NLRs) and other molecules related to the inflammasome pathway are upregulated in intracellular bacteria like Salmonella and Shigella species. Since Leishmania is an intracellular parasite, we analyzed the expression of some inflammasome pathway related components in Leishmania major-infected murine macrophages. For this, in this study we evaluated the expression levels of two NLRs (NALP3 and NAIP5), inflammasome adaptor molecule ASC, pro-inflammatory caspase-1, and pro-inflammatory cytokines IL-1β, IL-18. Semi-quantitative RT-PCR showed a significant increase of NALP3, IPAF, ASC, IL-1β, IL-18 mRNA level at 6 and 18 hours after L. major infection of macrophages compared to control ones, whereas, the expression level of NAIP5 was not significantly increased in infected macrophages.*

**Keyword:** NLRs, NALP3, IPAF, ASC, NAIP5, inflammasome, Leishmania major-infected murine macrophages

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## INTRODUCTION

Leishmaniasis is a complicated and important parasitic disease and for this, it has been of a great importance among the researches throughout the world (1, 2). It is able to survive inside macrophages through different mechanisms(3).The parasite generates nitric oxide (4), inhbits IL-12 production (5) and prevents NF-κBentry into the nucleus (6). It also has different mechanisms of pathogenesis (7,8).

Some intracellular microbes could activate innate immune response via NOD-like Receptors (NLRs). NLRs are a subset of pattern recognition receptors (PRRs) that are present in the cytosol and recognize endogenous or microbial moleculesand have an important role in detecting invading pathogens and initiating the innate immune response(9,10). Some NLRs, such as NLRP1, NLRP3 (NALP3), and NLRC4 (IPAF), oligomerize to formmultiproteininflammasome complexes. Once active, the inflammasome binds to pro-caspase-1, via its own

caspase activation and recruitment domain (CARD) or via the CARD ofthe adaptor protein ASC which it binds to during inflammasome formation (11, 12).Activation of caspase-1 through autoproteolytic maturation leads to the processing and secretion of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 (13).

By determining the expression of ASC adaptor molecule, IPAF, NAIP5, NALP3, IL-18, and IL-1B in mRNA level we can show if and which inflammsome pathway(s) is activated in Leishmania major-infected murine macrophages.

## MATERIALS AND METHODS

### 2-1. Cultivation of Parasite

Leishmania major, strain MRHO/IR/75/ER was purchased from Pasteur Institute of Iran (Tehran, Iran). The parasites were cultured in a biphasic NNN medium containing 250 IU/ml penicillin and 250 IU/ml of streptomycin to check the parasite growth. A fresh smear from liquid phase of biphasic culture after 3 days was prepared and observed under light microscope.

### 2-2. Infection of Macrophages

After parasite growth, the prostigotes were counted using ahemocytometer. As soon as the promastigotes count reached 2×10 6 /ml, they were transferred to culture tubes containing RPMI 1640 with 10 % fetal calf serum. To infect macrophages, metacyclic from of Leishmania major was used. At first the cultured macrophages were counted. Then a given concentration of them was prepared and transferred to a 6 chamber plate. After an overnight the cells were infected with the parasites in a ratio of 10:1 of parasite to cell. The culture medium then was placed at 37c. After 2 hours the supernatant of the medium was removed and the culture was washed 3 times by RPMI-1640 to exclude the free promastigotes. Then 5 ml of fresh medium was added to each chamber and placed in an incubator with the temperature of 37c for 2 hours.To eliminate free swimming promastigotes from the cultures, the supernatant was discarded and the macrophages were washed 3 times gently by RPMI 1640. Then, 5ml fresh RPMI 1640 was added to each culture chamber and incubated at 37c. After 6, 18 and 30 hrs, the infected macrophages harvested and kept at -20$^{\circ}$Cfor RNA extraction.

### 2-3. RNA Extraction and Reverse-Transcription

RNA extraction was performed by Tri-Pure Isolation Reagent (Roche, Germany) according to Manufacturer instruction. The extracted RNA was reverse-transcribed to cDNA by cDNA synthesize Kit(Fermentase, Life Science, Lithuania).

### 2-4. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed for ASC, (IPAF, NAIP5, NALP3), IL 1β, IL 18 by specific primers that were designed by Gen Runner and Primer Premier Softwares (Table 1). Hypoxanthine Phosphoribosyltransferase (HPRT) gene was used as the internal control. The intensity of obtained bands was determined by Kodak ID software. The data were analyzed by Kruskal-Wallis test.

**Table1: The sequence of the primers**

| Genes | Primers | Product size |
|---|---|---|
| ASC | F:5'-GCAACTGCGAGAAGGCTATG-3' | 311 bp |
| | R: 5'-AAGCATCCAGCACTCCGTC-3' | |
| NALP3 | F:5'-GCTAAGAAGGACCAGCCAGAGT-3' | 180 bp |
| | R: 5'-GAACCTGCTTCTCACATGTCGT-3' | |
| IPAF | F:5'-TTACTGTGAGCCCTTGGAGCA-3' | 395 bp |
| | R: 5'-TGCCAGACTCGCCTTCAATC-3' | |
| HPRT | F:5'-CGTCGTGATTAGCGATGATGAAC-3' | 609 bp |
| | R: 5'-TCACTAATGACACAAACGTGATTC-3' | |
| NAIP5 | F:5'-TTCACATCGAGAAGTTATCCATCCA-3' | 304bp |
| | R: 5'-AGCCTGGGCAAACTTTTCTGAC-3' | |
| IL-1β | F:5'-AGCTATGGCAACTGTTCCTGAAC-3' | 216 bp |
| | R: 5'-CTCCACAGCCACAATGAGTGATAC-3' | |
| IL-18 | F:5'-GCAGTAATACGGAATATAAATGACCAAG-3' | 188 bp |
| | R: 5'-GGTAGACATTTTACTATCCTTCACAGAGA-3' | |

## RESULTS

### 3-1. Primer Design

Primers were designed by Gen Runner and Primer Premier softwares. The sequence of the primers was as table 1.

### 3-2. RT-PCR Experiments Results

The following results were obtained from four independent series of macrophage cultures.
1-Expression analysis of ASC mRNA with semi-quantitative RT-PCR method in L. major infected and non-infected cells. The results show significant increase in ASC mRNA expression after 6 and 18 hours (Figure1).

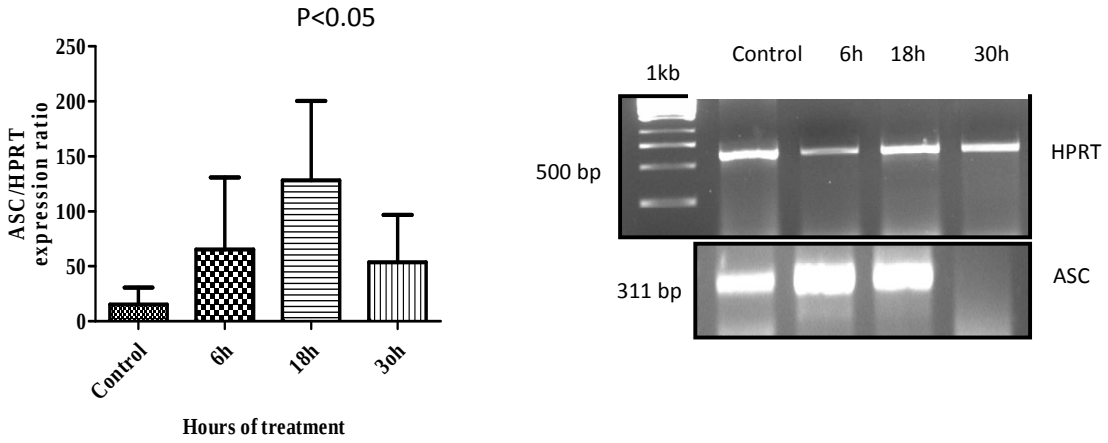


**Figure1: Comparison of the average of four separate experiments measuring mRNA ASC expression in cells infected with Leishmania major (6-18-30 hours) and non-infected cells.**

2- Expression analysis of IPAF mRNA with semi-quantitative RT-PCR method in L. major infected and non-infected cells. The results show significant increase in IPAF mRNA expression after 6 and 18 hours (Figure2).

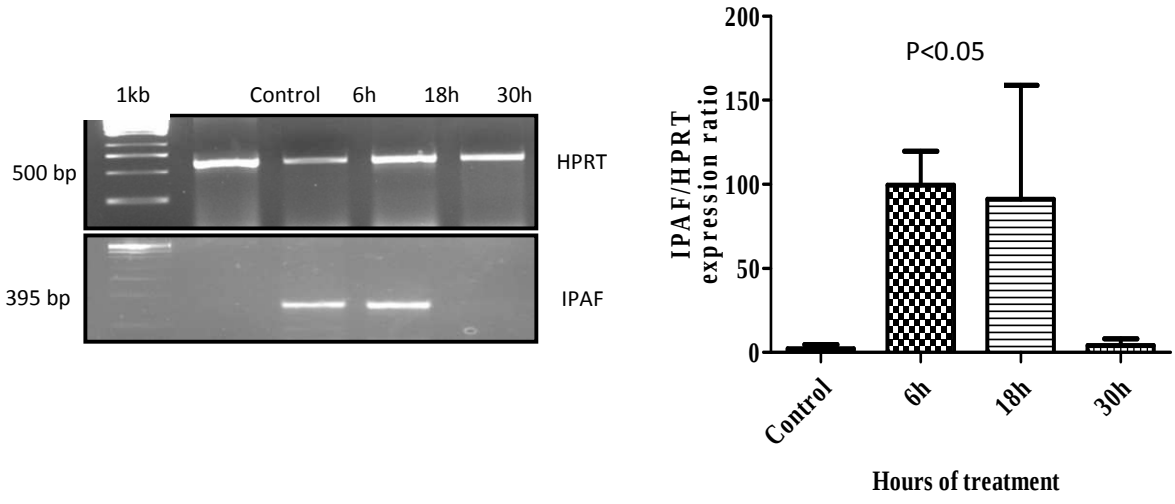


**Figure2: Comparison of the average of four separate experiments measuring mRNA IPAF expression in cells infected with Leishmania major (6-18-30 hours) and non-infected cells.**

3- Expression analysis of NALP3 mRNA with semi-quantitative RT-PCR method in L. major infected and non-infected cells. The results show significant increase in NALP3 mRNA expression after 6 and 18 hours (Figure3).

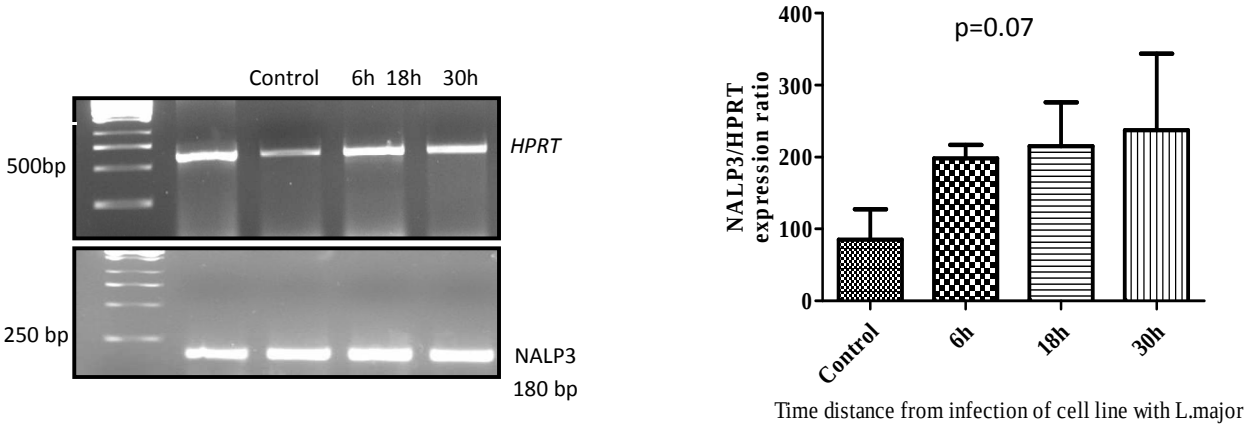


**Figure3: Comparison of the average of four separate experiments measuring mRNA NALP3 expression in cells infected with Leishmania major (6-18-30 hours) and non-infected cells.**

4- Expression analysis of NAIP5mRNA with semi-quantitative RT-PCR method in L. major infected and non-infected cells(Figure4).

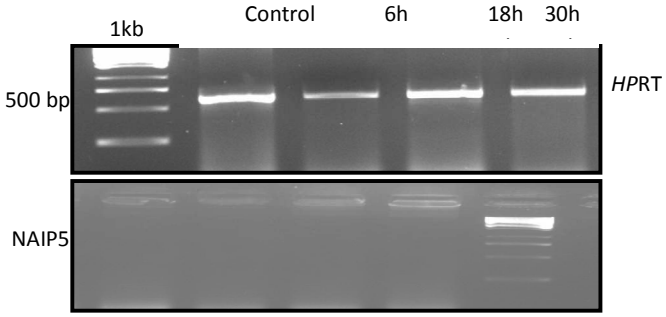


**Figure4: Comparison of the average of four separate experiments measuring mRNA NAIP5 expression in cells infected with Leishmania major (6-18-30 hours) and non-infected cells.**

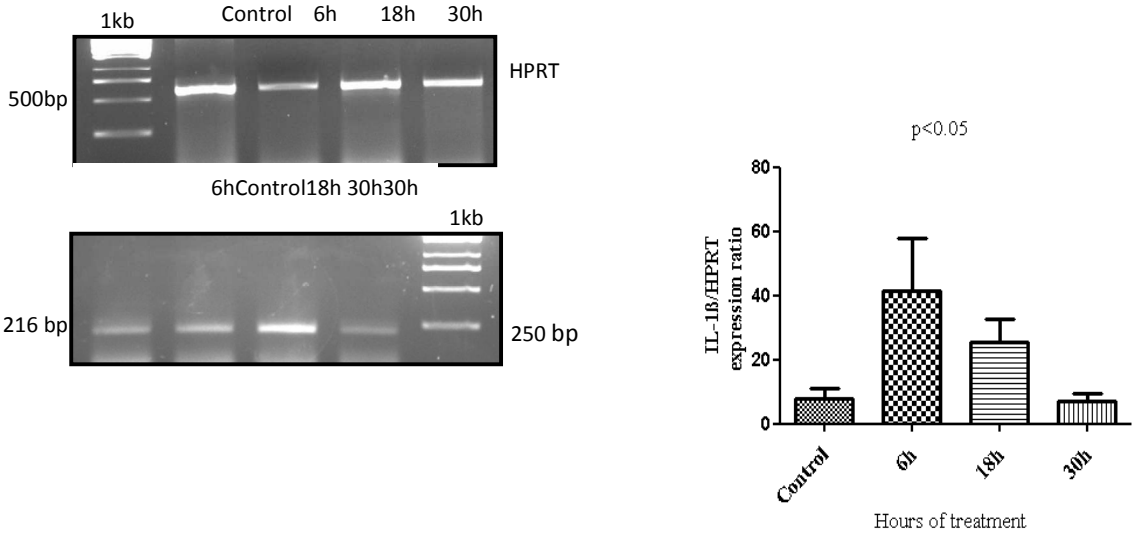


**Figure 5: Comparison of the average of four separate experiments measuring mRNA IL-1β expression in cells infected with Leishmania major (6-18-30 hours) and non-infected cells.**

5-Expression analysis of IL-1βmRNA with Semi-quantitative RT-PCR method in L. major infected and non-infected cells. The results show significant increase in NALP3 mRNA expression after 6 and 18 hours (Figure5).

6- Expression analysisofIL-18mRNA with Semi-quantitative RT-PCR method in L. major infected and non-infected cells. The results show significant increase in NALP3 mRNA expression after 6 hours (Figure6).

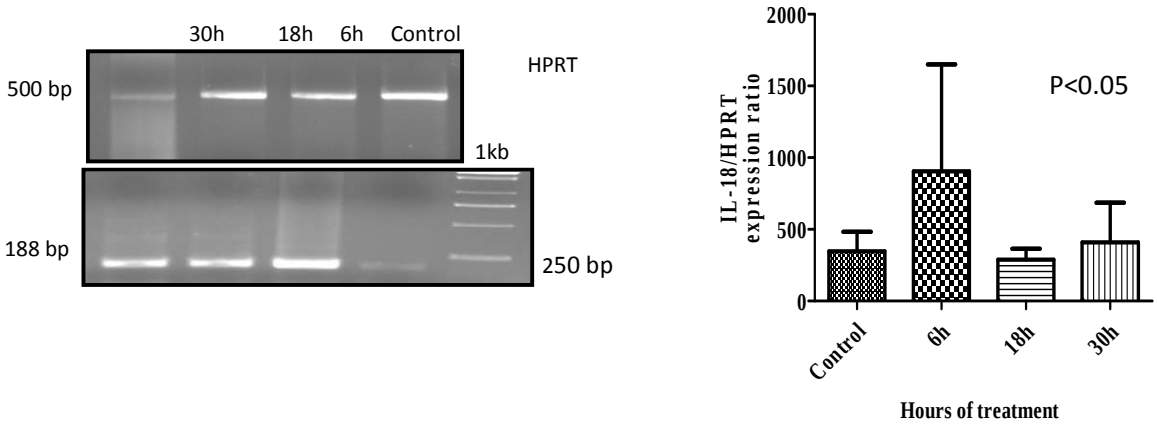


**Figure 6: Comparison of the average of four separate experiments measuring mRNA IL-18 expression in cells infected with Leishmania major (6-18-30 hours) and non-infected cells.**

## DISCUSSION

In the interaction between macrophage and parasite, there are various tricks for parasite survival within the macrophage (14).In this study, we demonstrated that some of inflammasome pathway related components such asNALP3,ASC, IPAF, IL-1β, IL-18are upregulated in Leishmania major-infected murine macrophages. The activity ofcaspase-1, also, in infected macrophages was higher than in non-infected cells (the results not shown here). We showed that the IPAFinflammosome pathway is active in Leishmania major-infected murine macrophages, but the results show thatNAIP5 inflammasome pathway is not active in these cells. Furthermore, caspase-1 activity in harvested cells and concentration of IL-1β in the supernatant of the cultured media were significantly increased in infected macrophages (the results not shown here). Altogether our results show the high expression of IPAF and NALP3inflammasome pathways in Leishmania major-infected murine macrophages. In vivo experiment in this field is highly recommended. Undoubtedly increasing knowledge about the mechanisms of activation and regulation of inflammosome pathway will expand our vision in the pathophysiology of chronic diseases and reveal new therapeutic targets.

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