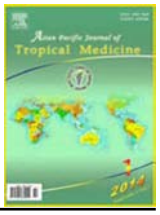
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading doi: 10.1016/S1995-7645(14)60205-4

Investigation of caspase-1 activity and interleukin-1β production in murine macrophage cell lines infected with *Leishmania major*

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ARTICLE INFO

Article history:
Article history:
Received 24 Apr 2014
Received in revised form 21 May 2014
Accepted 10 Jun 2014
Available online 13 Sep 2014

Article history:
Leishmania major
Caspase-1 activity
Interleukin-1β
Macrophage

ABSTRACT

Objective: To investigate the caspase-1 dependent inflammatory pathway activity and interleukin-1β (IL-1β) secretion in murine macrophage cell lines J774G8 infected with *Leishmania major* (*L. major*) using caspase-1 activity assay and ELISA.

Methods: Novy-MacNeal-Nicolle biphasic medium was applied to produce promastigote form of *L. major*. Metacyclic promastigotes in the stationary phase were applied to infect macrophage. Caspase-1 activity and IL-1β secretion were assessed by the CPP32/caspase-1 fluorometric protease assay and ELISA IL-1β kits, respectively, with time intervals of 6, 18 and 30 h.

Results: Our study showed an increase in caspase-1 activity and IL-1β secretion in infected samples compared to non-infected macrophages. The highest increase in IL-1β production was observed after 6 h of infection.

Conclusions: These results arise that the activation of inflammasome pathway could be one of the innate immunity pathways against *L. major*.

1. Introduction

Leishmaniasis is a parasitic disease that is found in parts of the tropics, subtropics, South American, Asia and Southern Europe. It has been estimated that there are 2 million new cases of leishmaniasis every year in the world, of which 1.5 million are categorized as cutaneous leishmaniasis and 0.5 million are visceral leishmaniasis[1-3]. This disease is caused by infection with *Leishmania* parasites that transmit to human beings through the bite of the female *Phlebotomine* sand flies. The parasites have a digenetic life cycle and exist in two distinct morphologies, the promastigote in sand fly vectors and the amastigote in mammalian hosts[4].

After the infection inside the mammalian hosts, *Leishmania* parasites infect the macrophages of reticuloendothelial tissue, differentiate into nonmotile amastigotes and then proliferate in the phagolysosomal vacuoles. In this process, *Leishmania* parasites in promastigote form are engulfed by macrophages but are resistant to proteolysis and degradation in the phagosome and transformed to amastigotes[5]. The *Leishmania* must endure harsh conditions inside the phagosome like the oxidative burst used by the macrophage to destroy foreign material inside the phagosome that consists of an attack by superoxide and hydroxyl radicals on the parasite. Accordingly, nitric oxide (NO) is a potent leishmanicidal factor in macrophages produced by the NO synthases, which is tightly regulated in response to infection by pathways that are not completely elucidated[6,7]. According to the study of Lima-Junior *et al.*[8], the NLRP3 inflammasome is activated in response to *Leishmania* infection and is important to restrict the parasite replication both in macrophages and *in vivo* as demonstrated through the

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Foundation Project: Supported by a grant from Sabzevar University of Medical Sciences (Grant No. 2012/25478).

infection of inflammasome-deficient mice with *Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania infantum* chagasi. Also inflammasome-driven interleukin-1β (IL-1β) production which facilitates host resistance to infection, as signaling through IL-1 receptor and MyD88, was necessary and sufficient to trigger inducible nitric oxide synthase (NOS2)-mediated production of NO[8].

Therefore, studies show that IL-1β is an important interleukin for host resistance to infection, particularly intracellular parasites which contributes to induce NOS2-mediated production of NO as a major host defense mechanism against *Leishmania* spp.[9-12]. On the other hand, studies showed that the Nod-like receptor (NLR) family is a significant protein complex which acts as a sensor to detect microorganism arrival by macrophage[13,14].

Certain NLRs regulate the assembly of NLRP3 inflammasome as a multimeric complex that contains active caspase-1, one of the key components of this complex with proteolytically enzyme activity that cleaves other proteins such as the precursor forms of inflammatory cytokines interleukin-1β and interleukin 18, into active mature peptide structures. Generally caspase-1 is activated when an external agent arrives into the macrophage and leads to the induction of IL-1β processing and secretion, which is transcriptionally regulated by IL-1β secretion when microbial components are sensed by pattern recognition receptors[8,15-17].

So, it is noteworthy that the activation of inflammasome leads to autonomous macrophage mechanisms that are culminated by the restriction of intracellular parasite replication. These processes involve the regulation of IFN-γ and the processing of IL-1β, which facilitate the expression of NOS2 required for NO-mediated restriction of *Leishmania* replication in macrophages[11,12].

Based on the importance of caspase-1 and IL-1β in inflammasome pathway and severity of leishmaniasis disease in humans, for the first time in this study, caspase-1 activity and IL-18 and IL-1β secretion levels were investigated and measured in macrophages infected with *Leishmania major* (*L. major*).

2. Materials and methods

2.1. Cell culture and infection process

To investigate the amount of caspase-1 enzyme in infected macrophages with *L. major*, *L. major* MRO/IR/57/ER was prepared from the Pasteur Institute in Tehran. Then the parasites were cultured into the Novy-MacNeal-Nicolle biphasic medium and supplemented with 250 IU/mL penicillin and 250 μg/mL of streptomycin. After 3 d, a new smear was extracted from the biphasic liquid phase and was evaluated by using microscope. Promastigotes were counted by using hemocytometer slide. After the number of parasites reached to 2×10^6/mL, promastigote were transferred to RPMI-1640 medium which containing 10% fetal calf serum at 25 °C until it reached to 7×10^7/mL. After 3 to 4 d, the procyclic forms of the parasite which are less infective turned into the metacyclic forms which are highly infective. Murine macrophage cell lines J774G8 were supplied from the Cell Bank of the Pasteur Institute of Iran. Macrophages were maintained into a flask which containing RPMI-1640, 15% fetal calf serum, 100 mg/mL streptomycin and 100 IU/mL penicillin and were incubated at 37 °C with 5% CO_2. After growth, the macrophages were trypsinated and cultured in 6 well plates about 1×10^6 cells/well at room temperature for 24 h. Promastigotes in the stationary phase which were converted to metacyclic used for macrophage infection with 10 parasites per cell and incubated at 37 °C for 2 h.

To remove free promastigotes from the culture medium, the supernatant of cell culture was discarded, and macrophages were gently washed three times by the RPMI-1640 medium. A total of 5 mL of the fresh RPMI-1640 medium were added to the previous samples and incubated for 6-18-30 h[18,19]. To detect the infected macrophages with intracellular parasites, the 400× Inverted Tissue Culture Microscope (tv0012000m USA) was used. Uninfected macrophages were cultured on similar manner.

2.2. Caspase-1 activity assay

To assess the activity of DEVD-dependent caspase, the CPP32/caspase-1 fluorometric protease assay kit (Biovision Company USA) was used. Enzyme activity is measured by using the substrate refraction (DEVD-AFC) by the enzyme. Crude substrate DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarine) emits blue light (400 nm), while in the present of caspase and its activity, the released AFC produces greenish yellow light (505 nm), which called fluorometer. The comparison between the infectious sample and the uninfected control, showed an increase in enzyme activity.

For caspase-1 activity assay, the reaction buffer 2× was divided into 300 μL volumes and then 3 μL of dithiothreitol was added to the reactant buffer (final concentration 10 mmol/L). For cell lysis, lysis buffer was added as much as three times of the cell amount, and homogenized. In the next stage, 50 μL of the suspension was transferred on ice, after 10 min, 50 mL of the reactant buffer containing dithiothreitol and 5 μL of DEVD-AFC substrate (final concentration of 50 mmol/L was added to it then was placed at 37 °C for 2 h. In the end, the fluorescent intensity was read by the fluorometer device (JASCO, FP-6200, Japan), in excitation wavelength of 400 nm and emission wavelength of 505 nm. The increase in enzyme activity was measured by comparing the results of infectious and control sample in different time.

2.3. ELISA test to assess the level of IL-1β in the cellculture supernatant

To evaluate IL-1β, ELISA IL-1β kit (RayBiotech, USA) was used. During the first step of incubation, 100 μL cell culture supernatant was added to each well and incubated for 1 d at -4 °C and then were washed and added to specific biotinylated monoclonal antibody against IL-1β. Then streptavidin-peroiydase enzyme was added and after incubation and was washed to remove unbound enzymes, suitable amount of enzyme substrate was added to create a colored product. The color intensity was directly proportional to the concentration of IL-1β in the samples.

3. Results

3.1. Comparison between caspase-1 activity in infected and non-infected murine macrophages

Mean values of caspase-1 activity were compared in infected and non-infected murine macrophage cell lines J774G8 by 1 mg of total cell culture lysate protein. Results showed an increase in caspase-1 activity in infected samples compared to non-infected (control) murine macrophages. According to these results, the inflammatory system of macrophage was stimulated by *L. major* ($P<0.05$) (Figure 1).

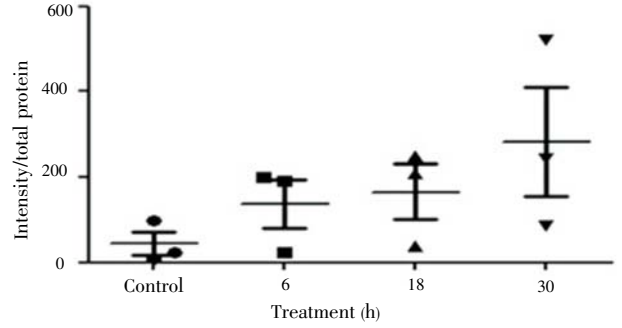

Figure 1. Caspase-1 activity after 6, 18, 30 h in macrophages after being infected by *L. major*.

3.2. Analysis of IL-1β secretion in in infected murine macrophages with L. major by ELISA.

The IL-1β secretion level was evaluated by ELISA test. Results showed an increase in the production of IL-1β in infected samples compared to the control (Figure 2).

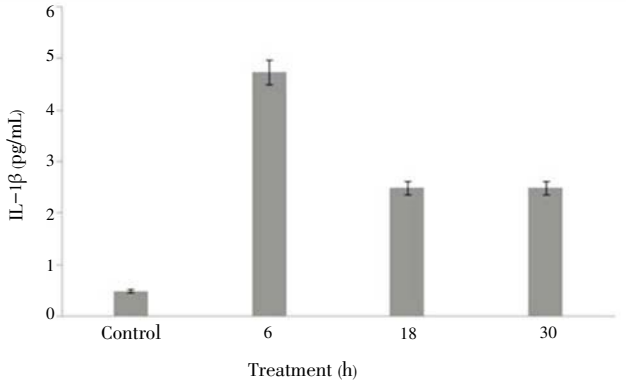

Figure 2. IL-1β secretion in infected macrophages after 6, 18 and 30 h measured by ELISA test.

In this period, an increase in the production of IL-1β can be observed by infected macrophages compared with the control. The highest increase in IL-1β production was seen after 6 h of infection.

4. Discussion

To overcome *Leishmania*, it is necessary to develop an appropriate immune response against the parasite. Some species of *Leishmania* are discovered to be immunologically dormant or silent which avoid recognition by innate immune receptors during the infection[8,11,12,20]. Our study revealed that caspase-1 and IL-1β are increased during the infection. This finding confirms that the activation of inflammasome pathway could be one of the innate immunity pathways against *L. major*. Activation of this pathway increases the production of IFN-β and γ which leads to the expression of NOS2, an essential enzyme producing NO that limits the parasite proliferation in macrophages[21-24]. There are also reports that measure the activity of beta signaling pathway to determine the intensity of disease in humans[25,26]. The results of *in vivo* studies showed that the mice with deficiency in caspase-1, IL-1β and IL-18 had a greater sensitivity to these parasites; therefore the activation of this pathway is necessary to inhibit the parasite proliferation. Regarding the overproduction of these products in infected samples compared to control, the important role of caspase-1 to resist against parasites is confirmed. Detecting the main pathways in host resistance to *Leishmania* is used as new therapeutic strategies through modifying inflammasome pathway to treat chronic diseases such as *Leishmania*[27,28]. Studies showed that *Shigella* needs caspase-1 and of IL-β and 18 productions to be virulent, mice deficient in production of capsase-1 are unable to develop inflammation[21,29]. Also, it was found that the activation of monocytogenes caspase-1 stimulates the production of IL-1β and IL-18[22,30]. Lara-Tejero *et al.* in 2006 examined the role of caspase-1 in the pathogenicity of *Salmonella typhimurium* in macrophages and measured the expression levels of IPAF, ASC, NALP3 genes at the level of mRNA in macrophages. In murine macrophages lacking ASC and NALP3, caspase-1 is activated, these results suggests that *Salmonella* can activate caspase-1 independent of ASC and NALP3, however it is not activated in macrophages lacking IPAF caspase. The significance of IPAF in activating caspase-1 was established[31].

Miao *et al.* showed that *Pseudomonas aeruginosa* activates caspase-1 in macrophages and causes the secretion of IL-1β and is 18[17]. They also proved that the T3ss flagellin antigen of the bacteria activates inflammasome pathway dependent IPAF and this path has been followed by activation of caspase-1 enzymes. The role of NAIP5 in the activation of caspase-1 was examined in this study, the caspase-1 is activated in macrophages lacking NAIP5[25]. Ozoren *et al.* in 2006 examined the distinct roles of TLR2 and ASC adapter molecules in IL-1β secretion in response to *Listeria monocytogenes*[30]. According to this study, the role of ASC in the host defense against the intracellular pathogen *Listeria monocytogene* is essential to secrete IL-1β and 18. However it is not necessary to produce IL-6, TNF-α, IFN-β in macrophages infected with *Listeria*. In macrophages deficient in ASC, caspase-1 activation does not occur, but activation of NF-κB, P38 is not affected[30].

The results imply the effect of *L. major* on the increased expression of inflammatory pathway-activating proteins and increased production of caspase-1 and IL-1β. Further research is recommended using other methods in terms of *in vivo* on mouse; the results can be generalized to humans after the experimental stage. Further *in vitro* studies and clinical trials are needed to confirm this hypothesis. Gaining much more information on the mechanisms of the activation of inflammatory pathways and the regulation of pathways will enhance the understanding about the pathology of some chronic inflammatory diseases as well as new therapeutic

goals[32,33].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was supported by a grant from Sabzevar University of Medical Sciences (Grant No. 2012/25478). We are grateful for all our colleagues and supporters for kind assistance.

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