IMMUNOREGULATION

2024. Vol 7

Research Paper

Pyrimethamine Enhances the Immune Responses of RAW264.7 Macrophages Against *Leishmania major*

Zahra Azimzadeh Tabrizi[1], Sara Soudi[1*], Ahmad Zavaran Hosseini[1], Mozhdeh Karimi[1]

1. Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

Use your device to scan and read the article online

Citation Azimzadeh Tabrizi Z, Soudi S, Zavaran Hosseini A, Karimi M. Pyrimethamine Enhances the Immune Responses of RAW264.7 Macrophages Against Leishmania major. Immunoregulation. 2024; 7:E15. http://dx.doi.org/10.32598/Immunoregulation.7.15

http://dx.doi.org/10.32598/Immunoregulation.7.15

Article info:
Received: 05 Sep 2024
Accepted: 15 Oct 2024
Available Online: 13 Dec 2024

Keywords:
Leishmania major, Macrophage, Immunometabolism, Pyrimethamine

ABSTRACT

Background: *Leishmania major*, an intracellular protozoan, is the primary cause of cutaneous leishmaniasis (CL). Although polyvalent antimony compounds are commonly used to treat CL, treatment failure is frequent. Immunometabolic reprogramming of macrophages—the main host cells for *L. major*—toward the M1 phenotype may inhibit parasite growth. Pyrimethamine, an antimalarial antibiotic, inhibits dihydrofolate reductase (DHFR), affecting both host and parasite metabolism. Since pyrimethamine interferes with the folate pathway and phagocytosis in *L. major*, this study investigated its effect on key immunometabolites in infected macrophages.

Materials and Methods: RAW264.7 macrophages were divided into six groups: Sham, *L. major*-infected, LPS-stimulated, pyrimethamine-treated (*L. major*+pyrimethamine, LPS+pyrimethamine), and pyrimethamine alone (5 µg/mL for 24 hours). Supernatants and cells were collected to measure lactate, nitric oxide (NO), reactive oxygen species (ROS), infection rate and index, as well as cytokine concentrations.

Results: Pyrimethamine significantly increased intracellular ROS and NO levels in *L. major*-infected macrophages ($P \leq 0.05$), correlating with a significant reduction in infection rate and index ($P \leq 0.05$). TNF-α production was also significantly elevated in pyrimethamine-treated groups. However, no significant change in lactate concentration was observed.

Conclusion: Pyrimethamine enhances the immunometabolic response of macrophages against *L. major* by increasing ROS, NO, and TNF-α levels. These changes contribute to a reduced infection rate and lower macrophage infection index, suggesting pyrimethamine's potential as an immunomodulatory agent in leishmaniasis treatment.

*** Corresponding Author:**
Sara Soudi, Associate Professor.
Address: *Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.*
Phone: *+98 (935) 2175558*
E-mail: *soudi@modares.ac.ir*

Introduction

Leishmaniasis is caused by intracellular protozoan species of the genus *Leishmania*, which are transmitted through the bites of sandflies, primarily those belonging to the genera Phlebotomus and Lutzomyia. This disease is endemic in several countries, particularly Brazil, Peru, Afghanistan, Iran, Saudi Arabia, and Syria [1]. There are three main clinical forms of leishmaniasis: Cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, and visceral leishmaniasis (the latter being systemic and fatal if left untreated) [2]. CL causes skin lesions that can range from mild papules to severe, ulcerative wounds [2]. In the Old World, it is mainly caused by *Leishmania tropica*, *Leishmania major*, and *Leishmania aethiopica*, while in the new world, the primary causative species include *Leishmania braziliensis*, *Leishmania mexicana*, and *Leishmania amazonensis* [3]. The cutaneous form is characterized by the development of skin lesions, such as papules and ulcers at the site of the sandfly bite [2].

Successful clearance of *L. major* from the infected host relies on the coordinated action of both innate and adaptive immune responses; however, these responses can be either protective or detrimental. Macrophages serve as the primary host cells for *Leishmania* parasites due to their long lifespan. They play a crucial role in determining the outcome of infection by either restricting or permitting parasite replication—thereby influencing the progression or resolution of leishmaniasis [4]. Studies using susceptible (BALB/c) and resistant (C57BL/6) mouse models of *L. major* infection have shown distinct immune responses. In C57BL/6 mice, IFN-γ produced by Th1 cells activates classically activated macrophages 1 (M1), leading to the production of nitric oxide (NO) and reactive oxygen species (ROS), which enhance parasite clearance. In contrast, in BALB/c mice, IL-4 produced by Th2 cells induces alternatively activated macrophages 2 (M2), which are characterized by the expression of arginase-1 and anti-inflammatory mediators [5].

Several methods have been developed to treat leishmaniasis. Although the most promising approach is vaccination, developing a vaccine against Leishmania has proven to be a major challenge. This difficulty arises primarily from limited understanding of the parasite's pathogenesis, the complexity of the immune response, and discrepancies between animal models and human disease. Consequently, there is currently no approved vaccine for human use, and antibiotics remain the primary approach for treatment and prevention of leishmaniasis [6, 7].

The effectiveness of antibiotics in combating infection depends not only on their direct antimicrobial activity but also on their immunomodulatory effects on host immune cells. Pentavalent antimonials, such as sodium stibogluconate and meglumine, remain the first line and standard treatment for all forms of leishmaniasis, particularly CL. Intralesional administration of antimonials has been introduced as an alternative to systemic delivery in order to reduce systemic side effects.

Other treatment options include amphotericin B, pentamidine, miltefosine, and paromomycin. However, these drugs come with several limitations, including the emergence of drug resistance, high treatment costs, incomplete parasite clearance, and significant side effects [3, 8]. Beyond their direct antimicrobial effects, antibiotics also influence immune cell metabolism. In recent years, increasing attention has been given to the link between the metabolic state of immune cells, particularly macrophages, and their immunological phenotype [9]. Metabolic mediators are not only sources of energy but can also modulate immune cell function. This dual role may be beneficial or detrimental to the host immune response. Therefore, an ideal antibiotic would not only eliminate the parasite but also exert favorable immunomodulatory effects [10].

Numerous studies have explored drugs targeting the folate metabolism pathway as a therapeutic strategy. One such drug is pyrimethamine, a diaminopyrimidine compound that acts as a folate antagonist essential for nucleic acid synthesis. It exerts its antimicrobial effect by inhibiting dihydrofolate reductase (DHFR), leading to tetrahydrofolate depletion and, consequently, inhibition of purine and pyrimidine biosynthesis [11]. While this enzymatic reaction is crucial for DNA synthesis in both bacteria and mammalian cells, the DHFR enzyme in bacteria is more sensitive to pyrimethamine than its mammalian counterpart [12]. This differential sensitivity underlies the drug's selective antimicrobial activity and has led to its use in treating bacterial infections [11]. Pyrimethamine is also widely used in the prevention and treatment of toxoplasmosis and malaria [13]. Importantly, pyrimethamine may also represent a promising therapeutic candidate for CL, given the high expression of DHFR in *L. major* [14]. The drug is available in oral form and has a relatively long half-life, remaining active in the body for several days [15]. Therefore, this research work investigates the effect of pyrimethamine on some of the key immunometabolic responses of *L. major*-ac-

tivated macrophages. The RAW264.7 cell line, derived from BALB/c mouse macrophages, was first stimulated with *L. major* and subsequently treated with various doses of pyrimethamine. The cytotoxic effects of Leishmania were then assessed using infection rate and index analysis. Macrophages and cell supernatants were also analyzed for NO and ROS production, lactate assay, and cytokine profile. While the individual techniques used (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT], flow cytometry, and enzyme-linked immunosorbent assay [ELISA]) are standard, the innovation lies in their integrative application and the specific research question they are used to answer. The key methodological innovation is the comprehensive and simultaneous profiling of both metabolic and immune parameters in a single experimental setup to elucidate the immunometabolic mechanism of an old drug against a parasitic infection. This study employed a controlled in vitro model to investigate the host-directed immunometabolic mechanisms of pyrimethamine, avoiding the confounding variables of animal models.

Materials and Methods

Culture and maintenance of the RAW264.7 cell line

The RAW264.7 cell line (first passage) was obtained from the Iranian Biological Resource Center (IBRC), Tehran, Iran. The cells were maintained and passaged using Dulbecco's Modified Eagle Medium (DMEM) complete medium (Gibco, UK). The DMEM complete medium consists of DMEM high glucose medium, L-glutamine (2 mM), sodium pyruvate, and is supplemented with 10% fetal bovine serum (FBS, Gibco, USA). After reaching 80-90% confluence, the cells were subcultured at a 1:3 ratio in T-25 flasks.

Drug preparation

Pyrimethamine (5,4-chlorophenyl-6-ethyl-2,4-pyrimidinediamine; CAS number: 58-14-0) was procured from a commercial source. A primary stock solution was prepared by dissolving the compound in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL. This stock was subsequently subjected to serial dilution in DMEM medium to generate working concentrations of 0.5, 2.5, 5, 50, 100, and 250 µg/mL for use in further assays.

Cytotoxicity evaluation of pyrimethamine

To determine the cytotoxic dose of pyrimethamine on RAW264.7 cells, an MTT test was performed. For this purpose, RAW264.7 cells (5×10^3 cells [50 µL]/well) in 96-well plates were treated with multiple doses of pyrimethamine (0.5, 2.5, 5, 50, 100, and 250 µg/mL) for 24, 48, and 72 hours. Then, 5 µL of MTT solution (5 mg/mL concentration) was added to each well for 3 hours. At the end of the incubation period, the supernatant from all wells was removed, and the developed formazan aggregates were dissolved in DMSO. The optical density was measured using a Labsystem Multiskan MS352 at 540 nm. The percent viability of cells in each group and the IC_{50} index for pyrimethamine were calculated using GraphPad Prism software, version 9 [16].

Annexin V-PI assay

Three doses of pyrimethamine (2.5, 5, and 10 µg/mL) were administered to cells, and the rates of apoptosis and necrosis of RAW264.7 cells were investigated. RAW264.7 cells (2×10^5 cells/well) received pyrimethamine (2.5, 5, and 10 µg/mL) for 24 h and then washed with 1×binding buffer ($CaCl_2$ [2.5 mM], NaCl [10 mM], and HEPES [10 mM]). Then, cells were resuspended in 1×PBS with FITC-tagged Annexin V and propidium iodide (PI) staining for 15 minutes at room temperature. The rates of apoptosis and necrosis in the cells were assessed using a BD FACSCalibur flow cytometer [17].

Parasite culture

L. major promastigotes (MRHO/IR/75/ER strain) were obtained from the Center for Research and Training in Skin Diseases and Leprosy, Tehran, Iran. The released promastigotes were cultured at 26 ˚C in RPMI 1640 medium supplemented with 10% FBS. The stationary phase of promastigotes was used to infect macrophages.

RAW 264.7 infection and experimental groups

RAW264.7 cells were divided into six experimental groups: 1) an untreated control group, 2) cells stimulated with 100 ng/mL LPS, 3) cells infected with *L. major* promastigotes at a 1:5 ratio, 4) LPS-stimulated cells treated with 5 µg/mL pyrimethamine, 5) *L. major*-infected cells treated with 5 µg/mL pyrimethamine, and 6) cells treated with 5 µg/mL pyrimethamine alone. For the infection, cells were seeded at 2×10^5 cells/well in a 6-well plate, incubated with stationary-phase *L. major* promastigotes (fixed in merthiolate for counting) for 24 hours at 37 ˚C with 5% CO_2. After incubation, the cells were washed and then incubated with either DMEM+10% FBS (control) or DMEM+10% FBS+5 µg/mL pyrimethamine (test group). Cell extracts and supernatants were collected from all groups for subsequent analysis.

Measurement of the infection rate and index

For the phagocytosis estimate, stationary-phase *L. major* promastigotes were enumerated using a Neubauer hemocytometer. The parasites were then added to the macrophages at a ratio of 5:1 (parasites to macrophages) and co-cultured for 24 hours at 37 ˚C in a 5% CO_2 atmosphere. Following the incubation period, the supernatant was carefully discarded. The adherent cells were subsequently fixed with methanol and stained with Giemsa stain for morphological analysis. The infection rate (percentage of infected macrophages) and infection index (mean number of amastigotes per infected macrophage) were determined by examining at least 100 macrophages per well under an inverted microscope [18].

Measurement of ROS production

To evaluate the ROS produced by RAW264.7 cells, we utilized 2,7-dichlorofluorescein diacetate (H2DCF-DA), a permeable and fluorogenic stain capable of measuring hydroxyl, peroxyl, and other intracellular ROS. For this purpose, RAW264.7 cells (2×10^5 cells/well) underwent treatment according to the study design for 24 hours at 37 ˚C in a 5% CO_2 atmosphere. The H2DCF-DA stain was dissolved in DMSO (10 µM) and subsequently diluted in DMEM culture medium. After incubation, the adherent cells were detached and incubated with 1 mL of H2DCF-DA for 45 minutes. Following washing with PBS, ROS production was evaluated using a BD FACSCalibur flow cytometer. The results were analyzed with FlowJo software [19].

Measurement of NO production

Similar to the ROS test, RAW264.7 cells (2×10^5 cells/well) were treated according to the study design for 48 hours. Subsequently, the supernatants from all six wells were collected to measure NO and other factors, such as lactate and cytokines. NO production was quantified using the Griess method, and various concentrations of NO were calculated based on the optical density (OD) at 540 nm of standard solutions of sodium nitrite, which were diluted and prepared in distilled water (CibBiotech Co., Iran).

Measurement of lactate production

The supernatants from all six groups were collected after 48 hours, and the amount of lactate production was measured using the ZellBio kit (GmbH, Germany), following the provided protocol. Subsequently, various concentrations of lactate were calculated according to the Equation 1:

1. $\frac{\text{OD sample-OD Blank}}{\text{OD standard-OD Blank}}\times 50$ mg/dL (standard concentration)=lactate concentration (mg/dL)

Cytokine measurement

The concentrations of TNF-α and IL-10 (pg/mL) in the supernatants of six groups after 48 hours were measured using the ELISA method with a commercial kit (R&D Systems, USA) following the manufacturer's protocol.

Statistical analysis

Statistical analysis of all experiments was conducted using GraphPad Prism software version 9.00. Data are expressed as the Mean±SD of at least three replicates. A one-way analysis of variance (ANOVA) was employed to clarify statistically significant variations among groups. Results with a $P\leq0.05$ were considered statistically significant.

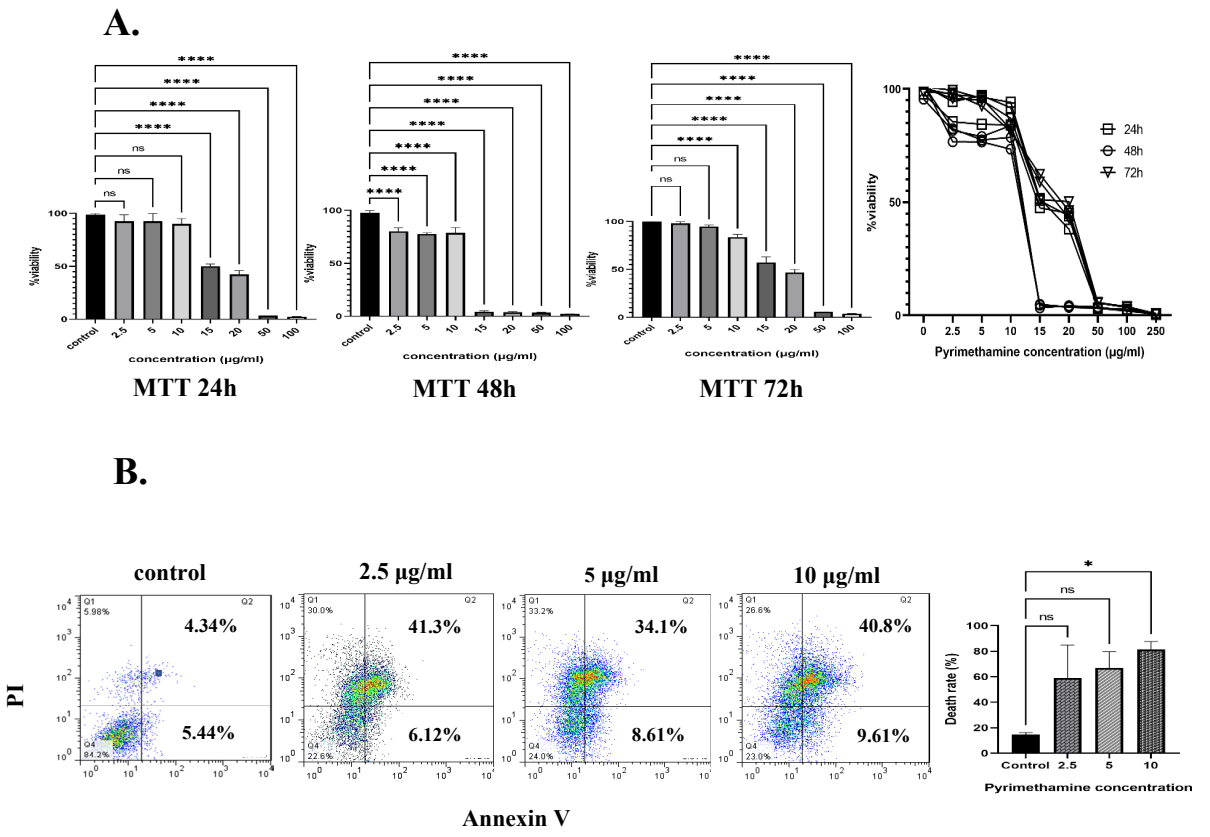
Results

Cell viability of RAW264.7 cells was reduced after pyrimethamine treatment

RAW264.7 cells (2×10^5 cells/well) were treated with 0.5, 2.5, 5, 10, 15, 20, 50, 100, and 250 µg/mL of Pyrimethamine for 24, 48, and 72 hours at 37 ˚C in a 5% CO_2 atmosphere. Cell metabolic activity and IC_{50} values were assessed using the MTT assay. The results for each time point are presented as the Mean±SD of viable cell counts in bar graphs, and the IC_{50} index was calculated to be 11.79 µg/mL using GraphPad Prism software, version 9. Pyrimethamine significantly suppressed the cell metabolic pathway at concentrations of 10 µg/mL and higher (Figure 1A). To validate the effects of pyrimethamine on cellular death, the cells were incubated with 2.5, 5, and 10 µg/mL of pyrimethamine for 24 hours to conduct the Annexin V-PI assay, and a significant increase in the percentage of cell death (apoptosis + necrosis) was observed following treatment with the concentration of 10 µg/mL (Figure 1B). The effective dose of pyrimethamine was defined as the lowest concentration exhibiting cytotoxicity, with 5 µg/mL considered the effective dose.

The infection rate and index of macrophages infected with *L. major* decreased after pyrimethamine treatment

Data are presented for controls (A), RAW264.7 cells infected with *L. major* (B), and RAW264.7 cells infected with *L. major* and treated with 5 µg/mL of pyrimethamine for 24 hours (C), observed using an inverted mi-



IMMUNOREGULATION

Figure 1. Effects of pyrimethamine on cell cytotoxicity

A) The viability percentage of RAW264.7 cells after exposure to different concentrations of pyrimethamine, B) The results of the Annexin V-PI assay after exposure to pyrimethamine

*P<0.05 vs controls (non-treated cells), **P<0.01 vs controls, ***P<0.001 vs controls.

Note: Data are presented as Mean±SD, with statistical significance determined by one-way ANOVA

croscope at 40x magnification. The mean infection rates were calculated as 81.53% for group B and 26.66% for group C. Additionally, the infection indices were 3.63 for group B and 1.16 for group C. Pyrimethamine-treated cells displayed a significant decrease in both the infection rate and index (Figure 2).

ROS production increased in macrophages infected with *L. major* after pyrimethamine treatment

RAW264.7 cells were infected with *L. major* and LPS and then treated with pyrimethamine (5 µg/mL). Intracellular ROS was measured using DCF-DA staining. Graphical representations of histograms with %ROS and mean fluorescent intensity (MFI) calculations were performed using FlowJo software. A one-way ANOVA was used to clarify significant differences among the groups (as shown in bar graphs). Following *L. major* infection, intracellular ROS and MFI were downregulated compared to the controls, but significantly increased after pyrimethamine treatment. The percentage of ROS and MFI in the RAW+LPS group, serving as the positive control, also increased after pyrimethamine treatment; however, this change was not statistically significant and was analyzed using FlowJo software, version 10, as illustrated in Figure 3.

NO production increased *L. major*-infected macrophages after pyrimethamine treatment

The mean levels of NO were higher in all groups treated with pyrimethamine than in the untreated groups. Additionally, there was a significant increase in NO levels (measured in µM) after pyrimethamine treatment in *L. major*-infected macrophages compared to the untreated group (Figure 4).

Lactate production decreased non-significantly after pyrimethamine treatment

As illustrated in the bar graph in Figure 5, the mean lactate levels in the RAW+LPS group were slightly higher

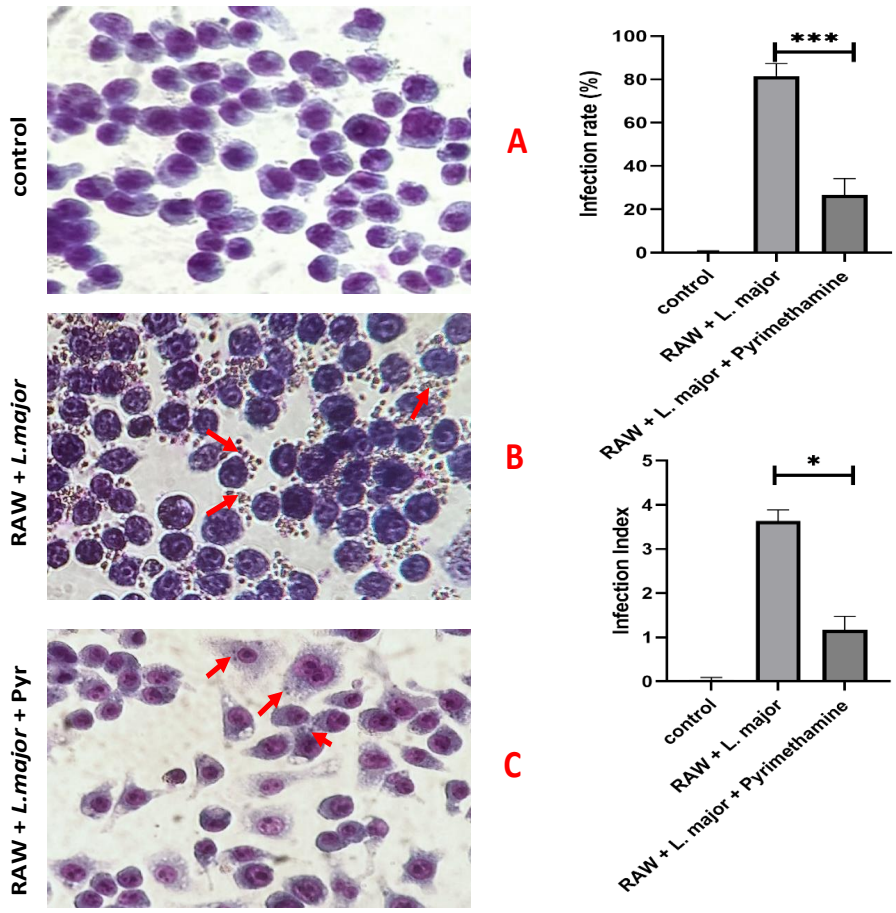


Figure 2. Effects of pyrimethamine on the infection rate and index of macrophages

IMMUNOREGULATION

*P<0.05 vs controls (non-treated cells), **P<0.01 vs controls, ***P<0.001 vs controls.

Note: (A, B, and C) Giemsa staining of *L. major*-infected cells in the three group. The red arrows indicate amastigotes that have been phagocytosed by macrophages. A one-way ANOVA was used to determine significant differences among the groups (bar graphs)

than those in the RAW+*L. major* group; however, no significant difference was observed. However, after treatment with pyrimethamine, lactate levels decreased non-significantly in both the RAW+*L. major* and RAW+LPS groups.

Pyrimethamine increased TNF-α levels and decreased IL-10 levels in macrophages infected with *L. major*

Regarding the results presented in Figure 6, the levels of TNF-α in the group infected with *L. major* were lower compared to the control group; however, after treatment with pyrimethamine (5 µg/mL), TNF-α levels significantly increased. Additionally, TNF-α levels in the control group also showed a significant increase following pyrimethamine treatment. Following the infection of macrophages with *L. major*, the IL-10 concentration increased, but this concentration decreased non-significantly after treatment with pyrimethamine.

Discussion

Parasitic diseases, such as leishmaniasis, pose a major global health burden with high mortality rates [8, 20]. CL, transmitted by sandflies, is widespread in Asia, including Iran; however, no human vaccine currently exists. Current drug treatments, including antimonials, amphotericin B, miltefosine, and paromomycin, are limited by high costs, side effects, and impractical administration routes [21]. Therefore, discovering new antileishmanial agents is urgent.

The investigation of a new group of antileishmanial compounds is essential. In addition to the direct effects that antibiotics have on killing the *Leishmania* parasite, they can also affect the metabolism of immune cells. The

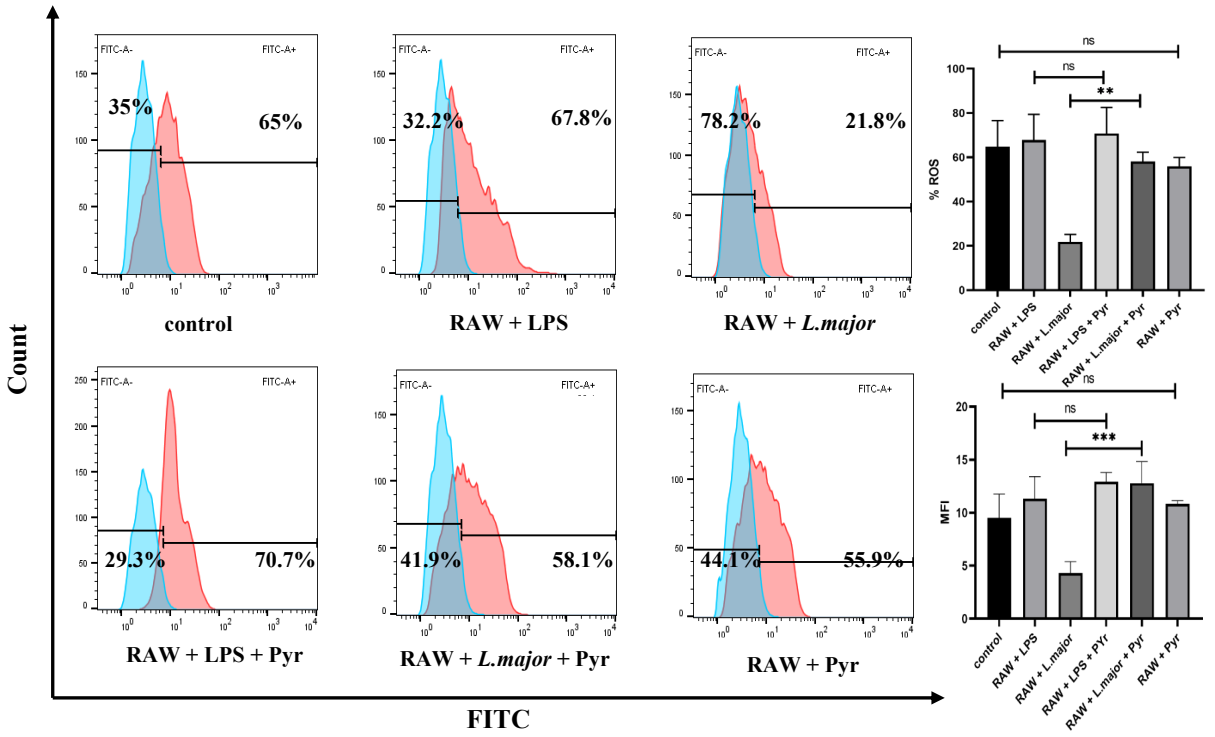


Figure 3. Measurement of ROS

IMMUNOREGULATION

*P<0.05 vs controls (non-treated cells), **P<0.01 vs controls, ***P<0.001 vs controls.

Note: The histogram results of ROS, along with MFI in the various groups, are presented with bar graphs. Data are presented as Mean±SD, with statistical significance determined by one-way ANOVA.

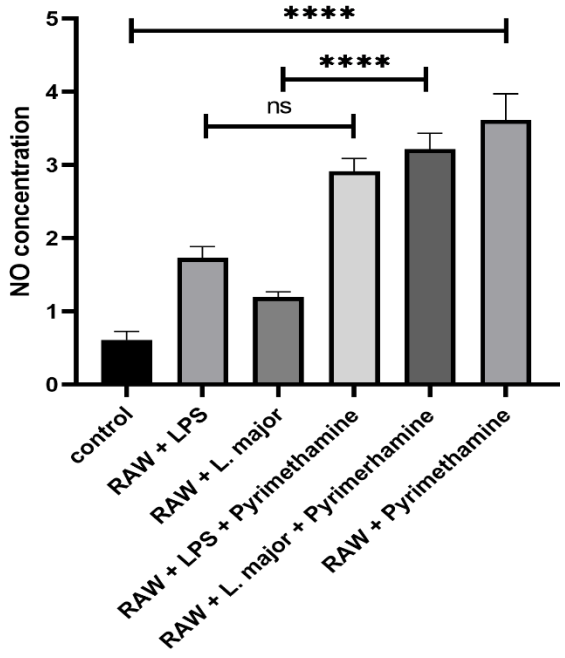
metabolism of activated immune cells not only meets their biosynthetic and bioenergetic needs but also acts as a switch to control their immune functions [22].

Therefore, an effective antibiotic, in addition to its direct effect, affects the metabolism and subsequently the function of the immune system. One such drug is pyrimethamine, which affects folate metabolism [15]. However, there are not many studies on the impact of this drug on the metabolic activity and function of immune cells in leishmaniasis. Given that macrophages are the primary host cells of the *Leishmania* parasite, they serve as an excellent model for illustrating the relationship between metabolism and function, as well as for investigating the effects of antibiotics on immunometabolic factors [23]. Thus, the core design of this study is a controlled in vitro (cell culture) experiment using the RAW264.7 mouse macrophage cell line. The design is structured to isolate and measure the specific effects of pyrimethamine on immunometabolic responses after *L. major* infection.

In the study conducted by Jung et al. in 2021, pyrimethamine enhanced autophagy and apoptosis in the chronic myeloid leukemia-derived KBM5 cell line by increasing the expression of STAT-5, JAK1/2, and Src at a dose of 0.25 µg/mL [24]. In addition, Giammarioli et al. demonstrated that pyrimethamine is capable of inducing apoptosis by increasing the cytochrome release and subsequently increasing the expression of caspase-9 in melanoma cell lines 8863 and 501 at a dose of 32 µmol/L [25]. Recently, Dong et al. demonstrated that pyrimethamine, at doses of 0.4 and 12.3 µM, reduced cell growth in the HCT116 and SW480 colorectal cancer cell lines, respectively [26].

The current study confirmed that the maximum cytotoxicity of pyrimethamine on the RAW264.7 cell line was 10 µg/mL (40 µmol/L), with an IC_{50} index of 11.79 µg/mL. Additionally, the results of the Annexin-PI assay demonstrated that a dosage of 10 µg/mL of pyrimethamine significantly increased apoptosis and necrosis in RAW264.7 macrophages compared to the control group. Based on the evidence and previous research, a concentration of 5 µg/mL (20 µmol/L) was selected for continued immunometabolic testing and further evaluation.

Lactate elevation as a result of aerobic glycolysis in M1 or classic macrophages leads to an excessive inflammatory response via increased expression of HIF-

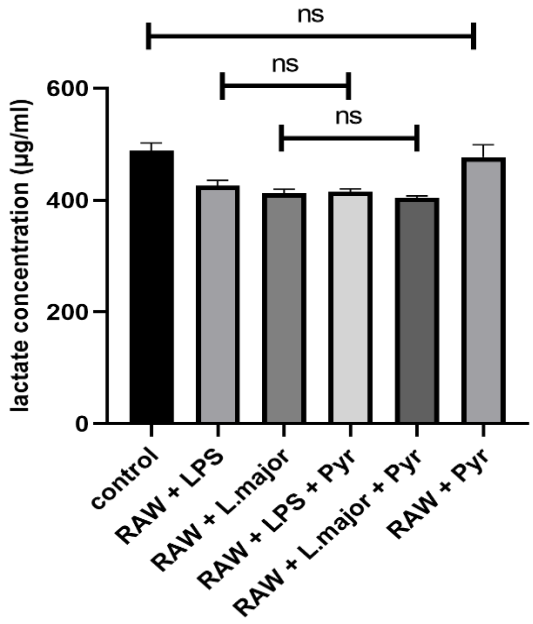


IMMUNOREGULATION

Figure 4. Measurement of NO levels in the experimental groups

*P<0.05 vs controls (non-treated cells), **P<0.01 vs controls, ***P<0.001 vs controls.

Note: The NO concentration in the supernatants of all groups is shown. The data display the Mean±SD of NO concentration in different study groups, analyzed using a one-way ANOVA (bar graph).



IMMUNOREGULATION

Figure 5. Lactate production in various experimental groups

*P<0.05 vs controls (non-treated cells), **P<0.01 vs controls, ***P<0.001 vs controls.

Note: The bar graph shows lactate levels (mg/dL) in different groups. Data are presented as Mean±SD and analyzed using one-way ANOVA.

1α, MMP-1, IL-1β, and IL-6 [27-29]. HIF-1α causes an upregulation in the production of the pro-inflammatory cytokine IL1β. This process occurs by binding HIF-1α to 300 base pairs before the transcription start locus of the IL1β gene, thereby increasing the transcription of this gene. These findings directly link glycolytic metabolism to the inflammatory function of macrophages [22, 30]. In contrast, M2 macrophages primarily rely on oxidative metabolism to provide energy for their long-term function [22]. In the present study, lactate concentration was reduced after treating infected macrophages with pyrimethamine; however, this reduction was not significant and may be related to its anti-inflammatory effects.

Gantt et al. demonstrated that after the phagocytosis of *Leishmania chagasi* promastigotes, both mouse and human macrophages are capable of producing substantial amounts of ROS and NO, which contribute to the elimination of the parasites [31]. In contrast, Filardy et al. found that following *L. major* infection, despite an increase in ROS and NO levels, as well as inflammatory responses, this reaction was not protective and instead facilitated parasite proliferation within macrophages [32]. Regarding the role of NO in protection against *L. major*, it was shown that reducing NO levels decreased the phagocytosis rate of *L. major* amastigotes, while the addition of NO to the in vitro environment enhanced this rate, confirming the beneficial effects of NO in killing *Leishmania* [33]. Leggoreta et al. reported that pyrimethamine leads to increased expression of superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes as a result of elevated oxidative stress in BALB/c mouse macrophages infected with plasmodium [34]. The results of our research indicated a correlation between increased oxidative stress and the infection rate. Our findings confirmed the role of oxidative stress in the elimination of *L. major* amastigotes, as evidenced by a significant decrease in both the infection rate and index. Furthermore, the results from the NO assay showed a significant increase in NO levels, leading us to conclude that this change is primarily due to the inflammatory effects of pyrimethamine on macrophages. In addition to the effects of pyrimethamine on oxidative stress, another factor contributing to the increase in ROS in RAW264.7 cells may be the high glucose concentration in the cell culture media. Suzuki et al. demonstrated that LPS-infected RAW264.7 cells exhibited elevated concentra-

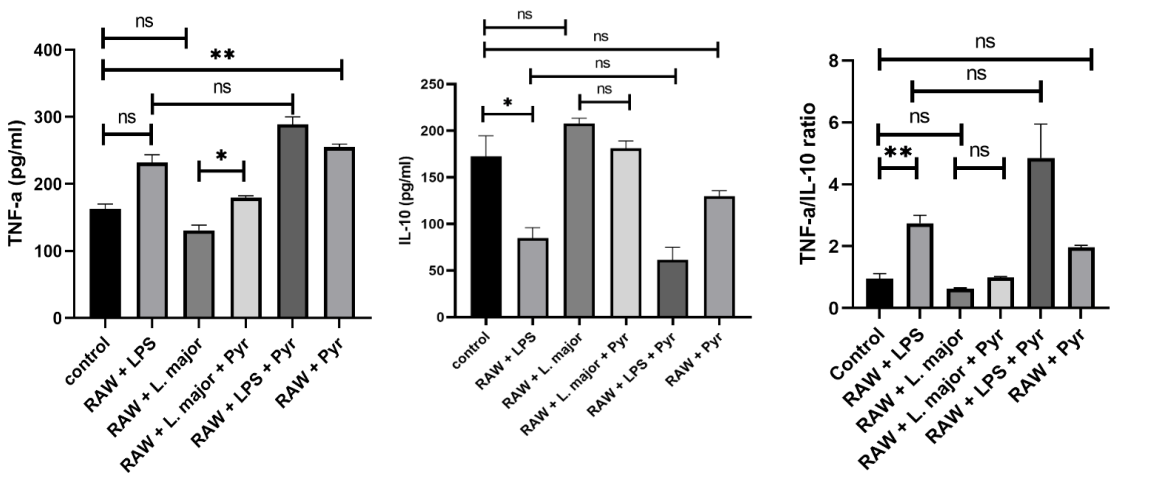


Figure 6. Cytokine production in the experimental groups

A and B) TNF-α and IL-10 levels in all groups (pg/mL), respectively, C) The TNF-α/IL-10 ratio in different groups.

*P<0.05 vs controls (non-treated cells), **P<0.01 vs controls, ***P<0.001 vs controls.

Note: Data are presented as Mean±SD and analyzed using one-way ANOVA.

tions of ROS and NO, as well as increased expression of NF-κB under hyperglycemic conditions [35].

The effects of inflammatory and anti-inflammatory responses in *L. major*-infected macrophages are significantly influenced by the distinct phenotypes of macrophages (M1 or M2). For instance, Vouldoukis et al. demonstrated that elevated levels of IL-10 and IL-4 in the M2 phenotype result in impaired clearance of *L. major* and *Leishmania infantum* [33]. Similarly, Liu et al. found that high expression of TNF-α and IFN-γ in M1 macrophages correlates with increased levels of iNOS and NO, which are crucial for the elimination of *Leishmania* [36]. Our study revealed that infected macrophages treated with pyrimethamine secreted elevated TNF-α levels, accompanied by decreased IL-10 levels compared to untreated groups. This was attributed to a significant increase in TNF-α concentration, which led to a significant decrease in the phagocytosis rate of *L. major* amastigotes.

Finally, the results of our study verified the inflammatory role of pyrimethamine in inducing oxidative stress and TNF-α secretion in macrophages reacting to *L. major* infection, as evidenced by a reduction in the infection rate and index of infected macrophages. However, pyrimethamine treatment did not result in a significant change in lactate concentrations. While our in vitro findings demonstrate pyrimethamine's ability to enhance anti-leishmanial responses in macrophages, these results must be interpreted cautiously due to the limitations of cell-line models. The absence of immune cellular crosstalk, tissue microenvironment, and systemic regulation underscores the need for validation in primary cells and in vivo models to establish physiological relevance in further studies.

Conclusion

In conclusion, our study on the immunometabolic responses of pyrimethamine in macrophages infected with *L. major* demonstrated that pyrimethamine increases oxidative stress and NO production, as well as the inflammatory cytokine TNF-α. This increase subsequently results in a reduction in the percentage of macrophages infected with *L. major*. Therefore, due to the direct effects of pyrimethamine on the elimination of *L. major* and its indirect effects on macrophage metabolism, it holds potential for use in macrophage polarization in future research.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

Funding

This research was supported by a grant from Tarbiat Modares University, Tehran, Iran.

Authors' contributions

Conceptualization, supervision, review, and editing: Sara Soudi; Methodology: Zahra Azimzadeh Tabrizi, Ahmad Zavaran Hosseini and Mozhdeh Karimi; Data curation and formal analysis: Zahra Azimzadeh Tabrizi; Writing the original draft: Zahra Azimzadeh Tabrizi, Ahmad Zavaran Hosseini, and Mozhdeh Karimi; Validation: All authors.

Conflicts of interest

The authors declared no conflict of interest.

Acknowledgements

The authors are grateful to the Factually of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

References

[1] Roatt BM, de Oliveira Cardoso JM, De Brito RCF, Coura-Vital W, de Oliveira Aguiar-Soares RD, Reis AB. Recent advances and new strategies on leishmaniasis treatment. Applied Microbiology and Biotechnology. 2020; 104(21):8965-77. [DOI:10.1007/s00253-020-10856-w] [PMID]

[2] Conceição-Silva F, Morgado FN. Leishmania Spp-host interaction: There is always an onset, but is there an end? Frontiers in Cellular and Infection Microbiology. 2019; 9:330. [DOI:10.3389/fcimb.2019.00330] [PMID]

[3] Sabzevari S, Teshnizi SH, Shokri A, Bahrami F, Kouhestani F. Cutaneous leishmaniasis in Iran: A systematic review and meta-analysis. Microbial Pathogenesis. 2021; 152:104721. [DOI:10.1016/j.micpath.2020.104721] [PMID]

[4] Matos AP, Viçosa AL, Ré MI, Ricci-Júnior E, Holandino C. A review of current treatments strategies based on paromomycin for leishmaniasis. Journal of Drug Delivery Science and Technology. 2020; 57:101664. [DOI:10.1016/j.jddst.2020.101664]

[5] Vellozo NS, Ribeiro-Gomes FL, Lopes MF. Shifting macrophage phenotypes in leishmaniasis. In: Kumar V, editor. Macrophages - Celebrating 140 Years of Discovery. IntechOpen; 2022. [DOI:10.5772/intechopen.105571]

[6] Ikeogu NM, Akaluka GN, Edechi CA, Salako ES, Onyilagha C, Barazandeh AF, et al. Leishmania immunity: advancing immunotherapy and vaccine development. Microorganisms. 2020; 8(8):1201. [DOI:10.3390/microorganisms8081201] [PMID]

[7] Abdellahi L, Iraji F, Mahmoudabadi A, Hejazi SH. Vaccination in Leishmaniasis: A review article. Iranian Biomedical Journal. 2022; 26(1):1-35. [DOI:10.52547/ibj.26.1.35] [PMID]

[8] Garza-Tovar TF, Sacriste-Hernández MI, Juárez-Durán ER, Arenas R. An overview of the treatment of cutaneous leishmaniasis. Faculty Reviews. 2020; 9:28. [DOI:10.12703/r/9-28] [PMID]

[9] Peters NC, Sacks DL. The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis. Cellular Microbiology. 2009; 11(9):1290-6. [DOI:10.1111/j.1462-5822.2009.01348.x] [PMID]

[10] Miller M, Singer M. Do antibiotics cause mitochondrial and immune cell dysfunction? A literature review. The Journal of Antimicrobial Chemotherapy. 2022; 77(5):1218-27. [DOI:10.1093/jac/dkac025] [PMID]

[11] De Carvalho PB, Ramos DC, Cotrim PC, Ferreira EI. Synthesis and in vitro evaluation of potential anti-leishmanial targeted drugs of pyrimethamine. Journal of Pharmaceutical Sciences. 2003; 92(10):2109-16. [DOI:10.1002/jps.10476] [PMID]

[12] Schweitzer BI, Dicker AP, Bertino JR. Dihydrofolate reductase as a therapeutic target. FASEB Journal. 1990; 4(8):2441-52. [DOI:10.1096/fasebj.4.8.2185970] [PMID]

[13] Beard EL Jr. The American Society of Health System Pharmacists. JONA'S Healthcare Law, Ethics and Regulation. 2001; 3(3):78-9. [DOI:10.1097/00128488-200109000-00003] [PMID]

[14] Adam I, Hagelnur AA. Artesunate plus sulfamethoxypyrazine/pyrimethamine for the treatment of cutaneous leishmaniasis: A double-blind, placebo-controlled clinical trial. International Journal of Antimicrobial Agents 2009; 34(4):380-1. [DOI:10.1016/j.ijantimicag.2009.03.013] [PMID]

[15] Sibley CH, Hyde JE, Sims PF, Plowe CV, Kublin JG, Mberu EK, et al. Pyrimethamine-sulfadoxine resistance in Plasmodium falciparum: What next? Trends in Parasitology. 2001; 17(12):582-8. [DOI:10.1016/S1471-4922(01)02085-2] [PMID]

[16] Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. In: Markossian S, Grossman A, Baskir H, Arkin M, Auld D, Austin C, et al, editors. Assay guidance manual. Bethesda: Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004. [PMID]

[17] Zhang G, Gurtu V, Kain SR, Yan G. Early detection of apoptosis using a fluorescent conjugate of annexin V. BioTechniques. 1997; 23(3):525-31. [DOI:10.2144/97233pf01] [PMID]

[18] Rahmani Y, Mohammadi-Yeganeh S, Yeganeh F, Koochaki A, Hoseini MH. A Simple technique for simultaneously quantifying cell death index and infection index on in vitro cell-based susceptibility assays. student research in translational medicine. 2024; 6. [DOI:10.22037/srtm.v6.46086]

[19] Gardiner B, Dougherty JA, Ponnalagu D, Singh H, Angelos M, Chen CA, et al. Measurement of oxidative stress markers in vitro using commercially available kits. 2020 Aug 9. In: Berliner LJ, Parinandi NL, editors. Measuring Oxidants and Oxidative Stress in Biological Systems. Cham (CH): Springer; 2020. [DOI:10.1007/978-3-030-47318-1_4] [PMID]

[20] Calvopiña M, Martinez L, Hashiguchi Y. Cutaneous leishmaniasis "chiclero's ulcer" in subtropical Ecuador. The American Journal of Tropical Medicine and Hygiene. 2013; 89(2):195-6. [DOI:10.4269/ajtmh.12-0690] [PMID]

[21] Kedzierski L. Leishmaniasis Vaccine: Where are we today? Journal of Global Infectious Diseases. 2010; 2(2):177-85. [DOI:10.4103/0974-777X.62881] [PMID]

[22] Galván-Peña S, O'Neill LA. Metabolic reprograming in macrophage polarization. Frontiers in Immunology. 2014; 5:420. [DOI:10.3389/fimmu.2014.00420] [PMID]

[23] Jha AK, Huang SC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity. 2015; 42(3):419-30. [DOI:10.1016/j.immuni.2015.02.005] [PMID]

[24] Jung YY, Kim C, Ha IJ, Lee SG, Lee J, Um JY, et al. Pyrimethamine modulates interplay between apoptosis and autophagy in chronic myelogenous leukemia cells. International Journal of Molecular Sciences. 2021; 22(15):8147. [DOI:10.3390/ijms22158147] [PMID]

[25] Giammarioli AM, Maselli A, Casagrande A, Gambardella L, Gallina A, Spada M, et al. Pyrimethamine induces apoptosis of melanoma cells via a caspase and cathepsin double-edged mechanism. Cancer Research. 2008; 68(13):5291-300. [DOI:10.1158/0008-5472.CAN-08-0222] [PMID]

[26] Dong H, Hu L, Li W, Shi M, He L, Wang C, et al. Pyrimethamine inhibits cell growth by inducing cell senescence and boosting CD8+ T-cell mediated cytotoxicity in colorectal cancer. Molecular Biology Reports. 2022; 49(6):4281-92. [DOI:10.1007/s11033-022-07262-y] [PMID]

[27] Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1: interaction between H-ras and hypoxia. Journal of Biological Chemistry. 2001; 276(12):9519-25. [DOI:10.1074/jbc.M010144200] [PMID]

[28] Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. The Journal of Biological Chemistry. 1996; 271(51):32529-37. [DOI:10.1074/jbc.271.51.32529] [PMID]

[29] Nareika A, He L, Game BA, Slate EH, Sanders JJ, London SD, et al. Sodium lactate increases LPS-stimulated MMP and cytokine expression in U937 histiocytes by enhancing AP-1 and NF-kappaB transcriptional activities. American Journal of Physiology. Endocrinology and Metabolism. 2005; 289(4):E534-42. [DOI:10.1152/ajpendo.00462.2004] [PMID]

[30] Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature. 2013; 496(7444):238-42. [DOI:10.1038/nature11986] [PMID]

[31] Gantt KR, Goldman TL, McCormick ML, Miller MA, Jeronimo SM, Nascimento ET, et al. Oxidative responses of human and murine macrophages during phagocytosis of Leishmania chagasi. Journal of Immunology. 2001; 167(2):893-901. [DOI:10.4049/jimmunol.167.2.893] [PMID]

[32] Filardy AA, Costa-da-Silva AC, Koeller CM, Guimarães-Pinto K, Ribeiro-Gomes FL, Lopes MF, et al. Infection with Leishmania major induces a cellular stress response in macrophages. PLoS One. 2014; 9(1):e85715. [DOI:10.1371/journal.pone.0085715] [PMID]

[33] Vouldoukis I, Bécherel PA, Riveros-Moreno V, Arock M, da Silva O, Debré P, et al. Interleukin-10 and interleukin-4 inhibit intracellular killing of Leishmania infantum and Leishmania major by human macrophages by decreasing nitric oxide generation. European Journal of Immunology. 1997; 27(4):860-5. [DOI:10.1002/eji.1830270409] [PMID]

[34] Ramos-Avila A, Ventura-Gallegos JL, Zentella-Dehesa A, Machuca-Rodríguez C, Moreno-Altamirano MM, Narváez V, et al. Immunomodulatory role of chloroquine and pyrimethamine in Plasmodium yoelii 17XL infected mice. Scandinavian Journal of Immunology. 2007 ; 65(1):54-62. [DOI:10.1111/j.1365-3083.2006.01869.x] [PMID]

[35] Suzuki T, Yamashita S, Hattori K, Matsuda N, Hattori Y. Impact of a long-term high-glucose environment on pro-inflammatory responses in macrophages stimulated with lipopolysaccharide. Naunyn-Schmiedeberg's Archives of Pharmacology. 2021; 394(10):2129-39. [DOI:10.1007/s00210-021-02137-8] [PMID]

[36] Liu D, Uzonna JE. The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. Frontiers in Cellular and Infection Microbiology. 2012; 2:83. [DOI:10.3389/fcimb.2012.00083]