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Expression of Cytokine Signaling Pathway Related Genes in *Leishmania*-infected Macrophages

Leishmania ile Enfekte Makrofajlarda Sitokin Sinyal Yolağı İlişkili Gen İfadesi

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ABSTRACT

Objective: Leishmaniasis, caused by protozoan parasites of the *Leishmania* spp., presents significant global health challenges, with visceral leishmaniasis (VL) and cutaneous leishmaniasis forms causing severe morbidity and mortality. Macrophages serve as primary host cells, where *Leishmania* spp. modulate immune 30 responses to ensure survival. Our study investigated gene expression changes in THP1-derived macrophages infected with *L. infantum* and *L. tropica* to elucidate host-pathogen interactions.
Methods: Macrophages were infected with stationary-phase promastigotes, and infection rates were confirmed via Giemsa staining. RNA was extracted, and real time-quantitative polymerase chain reaction was performed to analyze the expression of immune-related genes (*STAT1, STAT2, CCL4, IL23A, IL1R1, IL1RN*).
Results: Results demonstrated significant upregulation of STAT1 and STAT2, key mediators of the JAK-STAT pathway, in both infections, aligning with prior *in vivo* and *in vitro* studies. CCL4, a chemokine linked to macrophage recruitment, was also elevated, consistent with findings in VL and canine leishmaniasis. IL23A, associated with Th17 responses, showed increased expression, supporting its role in leishmanial immune modulation. Notably, IL1RN, an anti-inflammatory mediator, was upregulated, 40 suggesting a balancing mechanism to prevent excessive inflammation.
Conclusion: These findings highlight the complex interplay between pro- and anti-inflammatory responses during *Leishmania* infection and underscore potential targets for diagnostic and therapeutic strategies.
Keywords: *Leishmania*, macrophage, cytokine, JAK-STAT pathway

ÖZ

Amaç: *Leishmania* spp. protozoonlarının neden olduğu leishmaniasis, visseral leishmaniasis (VL) ve kutanöz leishmaniasis formlarıyla ciddi morbidite ve mortaliteye yol açarak küresel bir sağlık sorunu oluşturmaktadır. Makrofajlar, *Leishmania* spp.'nin hayatta kalmasını sağlamak için bağışıklık yanıtlarını modüle ettiği birincil konak hücrelerdir. Çalışmamızda, konak-patojen etkileşimlerini aydınlatmak amacıyla *L. infantum* ve *L. tropica* ile enfekte edilmiş THP-1 kaynaklı makrofajlardaki gen ekspresyon değişiklikleri incelenmiştir.
Yöntemler: Makrofajlar, durağan faz promastigotları ile enfekte edilmiş ve enfeksiyon 10 oranları Giemsa boyaması ile doğrulanmıştır. RNA izolasyonu sonrasında, bağışıklıkla ilişkili genlerin (*STAT1, STAT2, CCL4, IL23A, IL1R1, IL1RN*) ekspresyonunu analiz etmek için gerçek zamanlı-kantitatif polimeraz zincir reaksiyonu gerçekleştirilmiştir.
Bulgular: Sonuçlar, her iki enfeksiyonda da JAK-STAT yolunun önemli düzenleyicileri olan STAT1 ve STAT2'de belirgin bir artış olduğunu göstermiştir; bu bulgu, önceki *in vivo* ve *in vitro* çalışmalarla 15 uyumludur. Makrofaj birikmesiyle bağlantılı bir kemokin olan CCL4'ün de VL ve köpek leishmaniasisindeki bulgularla örtüşecek şekilde arttığı gözlemlenmiştir. Th17 yanıtlarıyla ilişkili IL23A'nın ekspresyonundaki artış, *Leishmania*'nın immün modülasyondaki rolünü desteklemektedir. Özellikle, anti-enflamatuvar bir mediatör olan IL1RN'deki yukarı regülasyon, aşırı enflamasyonu önlemeye yönelik bir denge mekanizmasına işaret etmektedir.
Sonuç: Bu bulgular, *Leishmania* enfeksiyonu sırasında pro- ve anti-enflamatuvar yanıtlar arasındaki karmaşık etkileşimi vurgulamakta ve tanısal/terapötik stratejiler için potansiyel hedefleri ortaya koymaktadır.
Anahtar Kelimeler: *Leishmania,* makrofaj, sitokin, JAK-STAT yolağı

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Leishmaniasis encompasses a spectrum of parasitic infections caused by different *Leishmania* spp., presenting diverse clinical challenges—from localized skin ulcers to fatal systemic infections affecting vital organs such as the liver, spleen, and bone marrow. The disease is endemic in over 90 tropical and subtropical countries across Asia, Africa, the Americas, and Europe (1). Classified as a neglected tropical disease, it affects more than 12 million people worldwide (2). Annually, 0.7-1.2 million new cases are reported, with approximately 350 million people at risk of infection (3).

Transmission occurs through the bite of infected female sand flies—Phlebotomus species in the Old World and Lutzomyia in the New World. To date, 21 *Leishmania* spp. and 30 sand fly vector species have been identified as capable of transmitting the disease to humans (4-5). The parasite exhibits a dimorphic life cycle, alternating between the motile promastigote stage in the vector and the non-motile amastigote stage in human hosts (2,6).

Clinically, leishmaniasis is categorized into four primary forms: Cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL), and post-kala-azar dermal leishmaniasis (PKDL). Among these, VL is the most severe, with the highest mortality rate due to its systemic nature (3). CL is primarily caused by *Leishmania aethiopica, L. infantum, L. tropica*, and *L. major* in the Old World, or *L. amazonensis, L. braziliensis, L. chagasi*, and *L. mexicana* in the New World (2). MCL is characterized by metastatic lesions that destroy mucous membranes and soft tissues in the mouth, nose, larynx, and pharynx. The main causative species—*L. braziliensis, L. panamensis, and L. guyanensis*—are predominantly found in Bolivia, Colombia, Ecuador, Peru, and Paraguay (7). In contrast, VL, also known as kala-azar, is a systemic infection caused by three *Leishmania* species: *L. chagasi, L. donovani,* and *L. infantum*.

Traditional diagnosis relies on detecting the pathogen via bone marrow aspirate smears or cultures (8). However, this method is invasive, often yields false negatives, and can lead to misdiagnosis. Moreover, VL lacks distinctive symptoms, making it difficult to distinguish from other illnesses, particularly in endemic regions (9). Consequently, there is a pressing need for improved diagnostic approaches.

Macrophages serve as the primary host cells for *Leishmania* spp. The parasites ensure their survival by altering macrophage metabolism (10). Studies using various host cell models have revealed gene expression changes during infection, shedding light on how *Leishmania* spp. modulates immune responses (11). Understanding gene expression patterns in *Leishmania* infection will improve our knowledge in disease biology which can positively impact both diagnosis and treatment. Therefore, in this study we investigated the changes in various gene expressions during *in vitro* macrophage infections of *L. infantum* and *L. tropica*.

METHODS

Monocyte Culture and Macrophage Differentiation

This study is designed as an *in vitro* experiment and does not require ethics committee approval. The human leukemia monocytic cell line THP-1 (kindly provided by Prof. Dr. Ayşe Nalbantsoy, Department of Bioengineering, Ege University) was maintained in 25 cm^2 sterile culture flasks. The cells were grown in RPMI-1640 medium (Biological Industries, USA) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, USA) and 1% penicillin/streptomycin (P/S, Gibco, USA). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere (Thermo Scientific, USA). To ensure optimal cell growth, the medium was refreshed every two days. THP-1 cells were seeded onto slides in 6-well plates at a density of 150,000 cells per well. To induce differentiation into macrophage-like cells and enhance their phagocytic activity, the cells were treated with 250 µg/mL PMA (Phorbol 12-myristate 13-acetate, Sigma, USA) and incubated for 24 hours prior to infection.

Promastigote Culture

Cryopreserved *L. infantum* (MCAN/TR/12/EP189) and *L. tropica* (MHOM/AZ/1974/SAF-K27) promastigotes were revived and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 27 °C. Initially, parasites were seeded at a density of 3 million per mL. Daily monitoring revealed logarithmic growth, with parasite counts reaching 8 million by day 3 and 15 million by day 5. Stationary-phase parasites were then harvested for macrophage infection.

Infecting Macrophages with *Leishmania*

Following differentiation of THP-1 cells into macrophages, the PMA-containing medium was removed and replaced with 1 mL of fresh medium containing *Leishmania* parasites at a concentration of 1,500,000 parasites/mL. The macrophage-parasite co-culture was then incubated for 24 hours at 37 °C. To determine the infection rate, Giemsa-stained slides were prepared, and the number of intracellular parasites per 100 macrophages was quantified via light microscopy.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from control and infected cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA). RNA concentration and purity were assessed using a Nanophotometer N60 (Implen, Germany), and samples were stored at -80 °C. For cDNA synthesis, a commercial kit (Biolabs, New England) was employed. Briefly, random primers were added to RNA samples, followed by incubation at 65 °C for 5 minutes in a thermocycler. The sample mix was then combined with reverse transcriptase enzyme and incubated for 1.5 hours to complete cDNA synthesis. The resulting cDNA was stored at -20 °C pending further analysis.

Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

All quantitative RT-PCR experiments were conducted using the Light Cycler 480 system (Roche, USA). Primer sequences are provided in Table 1. β-actin served as the endogenous control for normalization, with the following primer sequences: forward 5´-ATGATGATATCGCCGCGCTC-3´ and reverse 5´-TCGTCGCCCACATAGGAATC-3´. Reactions were performed in 10 µL volumes containing: 2 µL cDNA template, 5 µL SYBR Green I master mix (1×, Roche, USA), 500 nM of each primer, and 1 µL nuclease-free water. The thermal cycling protocol consisted of: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 10 s), and extension (72 °C, 10 s), with a final cooling step at 40 °C for 30 s. Amplification specificity was verified by melting curve analysis. Relative gene expression levels in *L. infantum*- and *L. tropica*-infected macrophages compared to uninfected controls were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Genes and primer sequences used in the study

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
STAT1	TGCTTGGATCAGCTGCAGAA	CCACCACAAACGAGCTCTGA
STAT2	GGGGCGCGAGGTTCTA	TGTCGAATGTCCACAGGCAG
CCL4	TGCTAGTAGCTGCCTTCTGC	CACTGGGATCAGCACAGACT
IL23A	GCTTCATGCCTCCCTACTGG	TGAGTGCCATCCTTGAGCTG
IL1R1	GAGCGGCAGGAATGTGACAA	CAAGGGGTCCAGCTTCTCAG
IL1RN	GACCTCCTGTCCTATGAGGC	GAGCATGAGGCTCAATGGGT

Statistical Analysis

All data were analyzed using GraphPad Prism version 9.2.0 for macOS (GraphPad Software, USA). Statistical significance was determined by One-Way ANOVA followed by Tukey's post hoc test for multiple comparisons. A p-value <0.05 was considered statistically significant. All experiments included three replicates.

RESULTS

Infection of THP-1 derived macrophages with *L. infantum* and *L. tropica*

Infectiveness of promastigotes were ensured as described before (12). Promastigotes from the stationary phase of the culture were used to infect macrophages. After 24 hours, co-culture was washed and stained with Giemsa, and observed under microscope. Approximately 50% of the macrophages were infected with at least one or more amastigotes. Figure 1 shows representative infected macrophages under 100X magnification.

Gene Expression Levels

Expression levels of six genes (*STAT1*, *STAT2*, *CCL4*, *IL23A*, *IL1R1*, and *IL1RN*) were investigated for three experimental groups (Macrophage, *L. infantum*, and *L. tropica*) via RT-qPCR. Total RNA was isolated via TRizol method. cDNA was synthesized from total RNA and used in qPCR. All genes exhibited significant increase in infection with both species. No significant difference was found between *L. infantum* and *L. tropica* infections for STAT1, STAT2, CCL4, and IL1RN. *L. infantum* infected macrophages exhibited higher expression of IL23A and IL1R1 compared to *L. tropica* infected macrophages. Figure 2 shows relative expression levels.

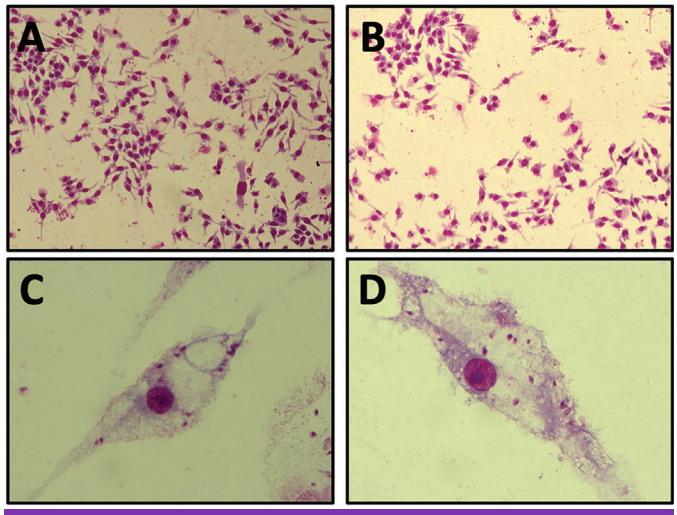

Figure 1. Infection of THP-1 derived macrophages. **A**) *L. infantum* amastigotes in macrophage (10X), **B**) *L. tropica* amastigotes in macrophage (10X), **C**) *L. infantum* amastigotes in macrophage (100X), **D**) *L. tropica* amastigotes in macrophage (100X)

DISCUSSION

In this study, THP-1 derived macrophages were infected with *L. infantum* and *L. tropica* species. Following the validation of infections under microscope, expression of several genes related to cytokine signaling were investigated via RT-qPCR. Namely, STAT1, STAT2, CCL4, IL23A, IL1R1, and IL1RN mRNA levels during *Leishmania* infections were measured.

Expression of STAT1 and STAT2, members of Signal Transducer and Activator of Transcription family were found to be significantly induced with the infection of both *L. infantum* and *L. tropica* species. The JAK-STAT pathway is a crucial signaling mechanism that mediates the effects of numerous cytokines. When this pathway malfunctions, it can contribute to the development and worsening of inflammatory and infectious diseases (13). In a study investigating cytokine pathway related gene expressions in skin lesions of *L. tropica* infected CL patients, authors found that five members of all STAT family, except STAT6 were significantly induced. STAT1 and STAT2 were among the most significantly increased members (14). In an *in vitro* study, Diotallevi et al. (15) infected U937-derived macrophages with *L. infantum, L. major,* and *L. tropica*. Following 24 and 48h, they performed RNA-Seq in order to reveal differentially expressed genes. They found and validated via qPCR that STAT1 expression is increased during infections by all three species (15). Our *in vitro* results correlate with both *in vivo* and *in vitro* data reported in the literature.

It was found that in VL induced by *L. donovani*, peripheral blood mononuclear cells (PBMC) expressed higher CCL4 compared to healthy controls (16). In canine VL caused by *L. infantum*, skin biopsy showed that the tissue is highly enriched with macrophages and CCL4 mRNA expression is significantly increased (17). It was also found upregulated in Diotallevi et al.'s (15) study mentioned above. Our results are consistent with the literature.

IL-23, a cytokine composed of two subunits, plays a key role in promoting Th17 cell differentiation (18). Microbial components—such as those from bacteria, viruses, fungi, and intracellular parasites—potently stimulate macrophages, monocytes, neutrophils, and dendritic cells to produce interleukin (IL)-23 (19). Hadifar et al. (14) found that intralesional expression of IL23A was significantly induced in *L. tropica* infected CL patients . Similarly, it was also found to be elevated in PBMC of CL patients (20). When cultured PBMC from PKDL patients were treated with total soluble *Leishmania* antigens, secreted IL23 was significantly

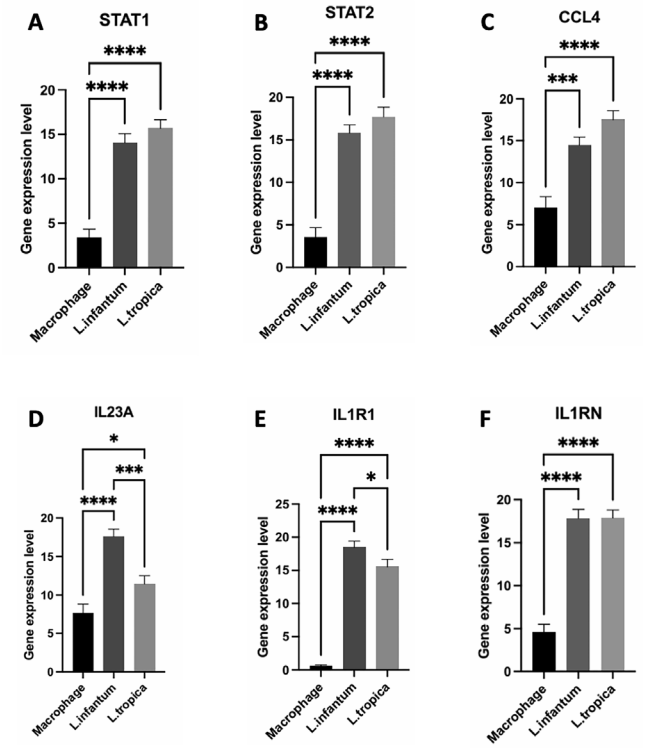

Figure 2. RT-qPCR results. Three experimental groups (uninfected Macrophage infected with *L. infantum*, and Macrophage infected with *L. tropica*) were investigated in terms of expression levels of six cytokine signaling-related genes. **A)** STAT1, **B)** STAT2, **C)** CCL4, **D)** IL23A, **E)** IL1R1, and **F)** IL1RN

**: p<0.05, ***: p<0.001, ****: p<0.0001, RT-qPCR: Real time-quantitative polymerase chain reaction*

increased compared to the PBMCs collected from healthy controls (21).

Fernandes et al. (11) infected THP-1 derived macrophages with three *Leishmania* spp. and compared the expression of long noncoding RNAs. They showed that IL1R1 was upregulated and coregulated with its antisense lncRNA in *L. infantum* infection.

Interestingly, along with pro-inflammatory cytokines, we found that anti-inflammatory IL1RN, antagonist of IL1 signaling, was also significantly upregulated in *L. infantum* and *L. tropica* infections. In an *in vivo* study, IL1RN KO mice manifested more severe disease progression (22), indicating that IL1R antagonist is important in restricting the immune response. Although parasite load was lower in IL1RN deficient mice, their survival rate was also decreased. Therefore, in the absence IL1R antagonist, the parasites are more efficiently cleared but at the same time the immune system is damaging the host. We found that IL1RN is upregulated in *Leishmania* infected macrophages *in vitro*.

Cytokines are pivotal in modulating the host's immune defense against *Leishmania* infection, influencing the balance between protective and pathological immune outcomes. Their biological effects are mediated through the activation of transcription factors, which translocate to the nucleus and regulate the expression of genes responsive to cytokine signaling. Understanding cytokine signaling and its regulation during leishmaniasis might result in better ways to both diagnose and treat the disease. Our *in vitro* results will contribute to literature in terms of revealing the role of cytokines in host defense against leishmaniasis.

CONCLUSION

This study demonstrates that *Leishmania* infection of macrophages triggers significant modulation of cytokine signaling pathways, characterized by upregulation of proinflammatory mediators alongside anti-inflammatory IL1RN. These findings underscore the dual role of host immune responses in controlling infection while preventing excessive inflammation. The consistent induction of JAK-STAT pathway genes across *L. infantum* and *L. tropica* infections highlights their potential as therapeutic targets. Further validation of these molecular signatures in clinical samples could advance diagnostic and immunomodulatory strategies for leishmaniasis.

*Ethics

Ethics Committee Approval: This study is designed as an *in vitro* experiment and does not require ethics committee approval.

Informed Consent: This study is designed as an *in vitro* experiment and does not require informed consent.

Footnotes

*Authorship Contributions

Concept: U.M., A.C., Design: U.M., H.A., A.C., Data Collection or Processing: U.M., H.A., C.M., A.C., Analysis or Interpretation: U.M., C.M., A.C., Literature Search: U.M., A.C., Writing: U.M., A.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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