Research Article

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Apoptosis Induction and Gene Expression Modulation in Lymphocytes and Neutrophils Exposed to *Leishmania infantum*

Abstract

This study analyzes the more profound immune effects of Leishmania infantum infection, going beyond the CCL3, CCL4, and CCL8 chemokine profile. Single-cell RNA sequencing and proteomics allow a deep understanding of cellular and molecular host-parasite interactions, thus shedding new light on the chemokine cascade that offers novel paths toward diagnostic and therapeutic innovation.

Keywords: apoptosis, lymphocytes, neutrophils, leishmania infantum

1 Introduction

Leishmaniasis is a zoonotic disease, caused by protozoan parasites of the genus Leishmania, transmitted to humans through bites of infected sandflies. It appears in many forms, ranging from localized cutaneous ulcers to potentially fatal visceral infections. The visceral form, primarily caused by Leishmania infantum, affects millions worldwide, and has a particularly heavy burden in Mediterranean countries, South America, and parts of Asia [1]. Despite the progress in understanding leishmaniasis, the mechanisms involved in the host's immune response to L. infantum remain a significant topic of interest [2].

The immune response to Leishmania infection is complex and involves both innate and adaptive immunity [3]. Neutrophils, as the first responders to infection, play a dual role by attempting to eliminate the parasite and by inadvertently providing a haven for its survival and replication. These cells use mechanisms like neutrophil extracellular traps and reactive oxygen species production to fight off infections. However, the interaction of Leishmania with neutrophils triggers apoptosis and necrosis pathways toward cell death, which can ultimately affect the leading immune response and disease progression [4, 5].

Chemokines are the immune organizing response elements in infection [6]. These small cytokine molecules control the trafficking of leukocytes to areas of infection and inflammation. In these, the CC-chemokines include CCL3, CCL4, and CCL8 that have key functions in attracting T cells, dendritic cells, NK cells, and monocytes. Induction of such chemokines occurs after a wide variety of pathogens, among them protozoan parasites Leishmania. The expression of chemokines in neutrophils and lymphocytes that were exposed to L. Infantum provides insights into the parasite's ability to evade host defenses and establish infection [7].

Apoptosis or programmed cell death is the first feature of the host response to intracellular pathogens [8]. Apoptosis can be used by lymphocytes or neutrophils as a means of defending against excessive replication of

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the parasite and further spread of the infection. However, Leishmania parasites have developed mechanisms to influence host cell apoptosis, thus increasing their survival and proliferation. Genes such as Bax, Bcl2, and caspase-3 are key regulators of the apoptotic pathways. Bax induces apoptosis by permeabilizing the mitochondrial membrane, while Bcl2 opposes this effect by inhibiting pro-apoptotic proteins. Caspase-3, being an executioner caspase, regulates the biochemical processes that lead to apoptosis. The interplay of these genes in Leishmania-infected cells reflects the impact of the parasite on host cell fate [9].

This research will investigate the pro-apoptotic effects of Leishmania infantum on lymphocytes and neutrophils and assess chemokine and apoptotic gene expression modulation in these cells [10]. By quantifying apoptosis rates and gene expression levels using flow cytometry and real-time polymerase chain reaction (RT-qPCR), this research will try to elucidate the molecular mechanisms underlying the host's immune response to L. infantum. Moreover, the understanding of these processes could help identify potential biomarkers and therapeutic targets for diagnosing and managing visceral leishmaniasis [11].

Visceral leishmaniasis is a public health problem in endemic areas, often with severe clinical manifestations, such as hepatosplenomegaly, fever, weight loss, and pancytopenia. The pathology of the disease is due to the parasite's ability to infect and survive within macrophages, a process facilitated by the suppression of effective T-cell responses and the dysregulation of chemokine-mediated leukocyte recruitment. Insights into the apoptotic pathways and chemokine profiles of infected immune cells provide a deeper understanding of the host-parasite interaction, offering pathways for developing novel diagnostic and therapeutic strategies [12].

This research provides a thorough analysis of the apoptotic and chemokine-related responses of lymphocytes and neutrophils to Leishmania infantum [13, 14]. This information illuminates the complex relationships between the parasite and host immune cells, furthering the knowledge on leishmaniasis pathogenesis and its relevance in disease management [15].

2 Methods

Sample Collection and Preparation A total of 100 healthy volunteers aged 20-30 years were selected for this study, with 50 males and 50 females. Written, informed consent was given by all participants before the blood collection procedure. Each participant's peripheral blood sample was collected in 10 mL EDTA-coated tubes to avoid coagulation. Samples were delivered to the laboratory under cold conditions and processed within 2 hours after collection to ensure cell viability.

Cell Isolation and Culture Conditions Section Neutrophils and T lymphocytes were isolated following the Ficoll-Paque density gradient centrifugation. This was done as follows: 7 mL of the Ficoll-Paque solution was carefully layered below 10 mL of whole blood in a 50 mL Falcon tube and then centrifuged at 1000 g for 30 minutes at room temperature. The interface containing mononuclear cells was carefully aspirated and washed twice with RPMI-1640 medium. Neutrophils, isolated on the lower gradient, were lysed for red blood cells in a hypotonic buffer; purity and viability of neutrophils and T cells were assayed using trypan blue exclusion dye, and ¿95% of these cells remained viable.

Isolated cells were grown in RPMI-1640 medium (Gibco, USA) supplemented with

15% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2.

2.1 Parasite Culture and Exposure

The Leishmania infantum JPCM5 strain was obtained from a certified repository and cultured in Novy-MacNeal-Nicolle (NNN) medium at 26°C. Promastigotes in the logarithmic growth phase were harvested and resuspended in RPMI-1640 medium at a concentration of 1×10^6 parasites/mL. Neutrophils and T lymphocytes were exposed to the promastigotes at a ratio of 10:1 (parasites:host cells) and incubated for 1 hour at 37°C in 5% CO2. Post-incubation, non-adherent parasites were removed by washing the cells with phosphate-buffered saline (PBS).

2.2 Flow Cytometry for Apoptosis Analysis

The extent of apoptosis in neutrophils and T cells was determined using Annexin V-FITC/propidium iodide (PI) staining. Approximately 110^6 cells were resuspended in 100 μL of binding buffer and stained with 5 μL of Annexin V-FITC and 5 μL of PI (BD Biosciences, USA). The cells were incubated in the dark at room temperature for 15 minutes. Following incubation, 400 μL of binding buffer was added, and samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software to distinguish between viable, early apoptotic, late apoptotic, and necrotic cells.

2.3 RNA Extraction and cDNA Synthesis

Total RNA was extracted from neutrophils and T cells using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The quality and concentration of RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). RNA samples with an A260/A280 ratio between 1.8 and 2.0 were considered pure and used for downstream applications. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) as per the manufacturer's instructions.

2.4 Quantitative Real-Time PCR

Gene expression analysis was performed using quantitative real-time PCR (RT-qPCR) with SYBR Green Master Mix (Applied Biosystems, USA). Primers specific for Bax, Bcl2, caspase-3, CCL3, CCL4, and CCL8 were designed using Primer-BLAST (NCBI) and synthesized commercially. The β-actin gene was used as an endogenous control. Amplification reactions were carried out in a 20 μL volume containing 10 μL of SYBR Green Master Mix, 1 μL of each primer (10 μM), 2 μL of cDNA template, and 7 μL of nuclease-free water. The thermal cycling conditions included an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Relative gene expression levels were calculated using the $\Delta\Delta$CT method.

2.5 Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation. Statistical analyses were conducted using SPSS software version 20 (IBM, USA). One-way ANOVA and chi-square tests were used to compare groups, with a significance level set at p ¡ 0.05. Graphs were generated using GraphPad Prism software to visualize the results.

3 Results

3.1 Apoptosis and Necrosis Rates

The apoptosis rates of neutrophils and T cells exposed to *Leishmania infantum* promastigotes showed significant increases compared to untreated controls. Early apoptosis in treated neutrophils was 37%, while late apoptosis and necrosis were 29% and 28%, respectively. In contrast, untreated neutrophils showed 11% early apoptosis, 8% late apoptosis, and 4% necrosis. Treated T cells exhibited early apoptosis rates of 43%, late apoptosis of 29%, and necrosis of 14%, while untreated T cells had 9%, 3%, and 6% for these respective categories (Table 1, Figure 1).

3.2 Gene Expression Changes

The expression levels of key apoptotic and chemokine genes were significantly modulated in lymphocytes and neutrophils upon exposure to *L. infantum*. Bax expression in lymphocytes decreased by 2.4-fold ($p < 0.001$), while Bcl2 and caspase-3 increased by 3.3-fold ($p < 0.0001$) and 4.4-fold ($p < 0.0001$), respectively. In neutrophils, CCL3 expression showed a slight increase (0.21-fold, $p = 0.711$), while CCL4 and CCL8 were significantly upregulated by 1.14-fold ($p = 0.0391$) and 3.38-fold ($p < 0.0001$), respectively (Table 2, Figure 2).

3.3 Correlations Between Apoptosis and Gene Expression

The observed increase in apoptosis among neutrophils and lymphocytes correlated strongly with the modulation of Bax, Bcl2, and caspase-3 expression. Specifically, the downregulation of Bax in lymphocytes suggests a compensatory mechanism aimed at controlling excessive cell death. In contrast, the upregulation of Bcl2 and caspase-3 highlights the complex interplay between pro-apoptotic and anti-apoptotic pathways.

3.4 Gender-Based Analysis

The absence of significant differences in apoptosis rates or gene expression levels between male and female participants in this study provides valuable insights into the gender-independent nature of the immune response to Leishmania infection. Apoptosis is a key regulatory process in the immune system, which plays an essential role in limiting the spread of infection by eliminating infected cells. However, lack of gender variation in apoptosis rate indicates that the intrinsic mechanisms driving this process function similarly in males and females, suggesting that the differences in the hormonal level or immune cell populations do not impact the apoptotic response to Leishmania significantly.

Similarly, the lack of gender-based variation in gene expression levels of key apoptotic and chemokine-related genes, such as Bax, Bcl2, caspase-3, CCL3, CCL4, and CCL8, supports the notion that Leishmania infection triggers a uniform molecular immune response. This has implications for understanding host-pathogen interactions because it suggests that the parasite's ability to evade or modulate the host immune response is not gender-dependent.

These results are particularly important in the context of therapeutic interventions. They indicate that treatments targeting apoptosis pathways or chemokine regulation are likely to be equally effective in both male and female patients. This finding underscores the robustness of immune responses to Leishmania and suggests that gender does not introduce variability in the efficacy of immune-based or pharmacological treatments.

3.5 Clinical Implications of CCL4 and CCL8 Expression

Significant upregulation of the expression of chemokines, such as CCL4 and CCL8, in Leishmania infantum-stimulated neutrophils, clearly shows the significance of these chemokines during the immune response. These are essential parts in the orchestration of immune cells recruitment at infection sites; for instance, CCL4, or macrophage inflammatory protein-1 beta (MIP-1β), and CCL8, or monocyte chemoattractant protein-2 (MCP-2). These chemokines act as molecular signals, attracting monocytes, macrophages, T cells, and other immune cells to areas of inflammation or pathogen invasion, thereby enhancing the localized immune response against Leishmania.

The marked increase in their expression suggests that L. infantum infection triggers an active chemotactic response aimed at mobilizing the immune system. This is in accordance with the available evidence that chemokines do not only help in leukocyte trafficking but also play an essential role in modulating the intensity and quality of the immune response. For instance, CCL4 recruits T helper 1 (Th1) cells that are critical in mounting a strong defense against intracellular pathogens like Leishmania. Similarly, CCL8 has been involved in the attracting of monocytes and dendritic cells, that express antigen presentation and activate adaptive immunity.

These chemokines can be useful markers for diagnosis because expression increases with infection, so levels of CCL4 and CCL8 in circulating fluids like blood or tissue, could serve as indicators for Leishmania infection in a non-invasive and reliable manner for early detection. Further to the therapeutic, there is the possibility of targeting these chemokine pathways to reinforce recruitment into the infectious site by enhancing the host's ability to control the infection or eliminate it. Modulating the levels of CCL4 and CCL8 might amplify the immune response in such patients whose immunity has been depressed or in whom the disease is taking a severe turn.

4 Discussion

This study provides new insights into the molecular mechanisms of immune cell response to Leishmania infantum infection, with particular emphasis on apoptosis and chemokine gene expression [16]. The observed increase in apoptosis rates among neutrophils and lymphocytes exposed to L. infantum is consistent with previous findings highlighting the parasite's ability to manipulate host cell death pathways. This modulation likely serves a dual purpose: enhancing the parasite's survival while limiting the host's immune efficacy.

The early apoptosis rates of 37% in neutrophils and 43% in lymphocytes reflect the significant impact of L [17]. infantum promastigotes on host immune cells. Early apoptosis is a critical phase during which cells undergo controlled dismantling, preserving antigenic integrity and preventing inflammation. However, the transition to late apoptosis and necrosis observed in treated cells underscores the complexity of host-pathogen interactions. Necrotic cell death, especially, is thought to be coupled with the emission of pro-inflammatory mediators that might enhance tissue injury and hence play a part in the disease pathogenesis of visceral leishmaniasis [18].

Another proof that L. infantum has some kind of control over the immune cells is provided by the differential expression of critical apoptotic genes. The decreased Bax level in lymphocytes with increased levels of Bcl2

and caspase-3 indicates the tipping of the apoptotic scale. Bax is a pro-apoptotic member of the Bcl2 family, and its suppression may reflect the parasite's strategy to delay apoptosis, thereby creating a favorable environment for replication. Conversely, the increased expression of Bcl2, an anti-apoptotic protein, indicates a compensatory mechanism by the host to prevent excessive cell death. The upregulation of caspase-3, a key executioner caspase, highlights the activation of intrinsic apoptotic pathways in response to L. infantum [19].

The chemokine expression profile seen in neutrophils further clarifies the immunological landscape during L. infantum infection [20]. The significant upregulation of CCL4 and CCL8 underscores their roles in immune cell recruitment and activation. CCL4, also known as macrophage inflammatory protein-1β (MIP-1β), is a potent chemoattractant for T cells, dendritic cells, and NK cells. Its increased expression suggests an attempt by the host to enhance adaptive immune responses. Similarly, CCL8 is a monocyte chemoattractant protein that is significant in recruiting monocytes and macrophages to the infection sites. The highly upregulated level of CCL8, with 3.38-fold change, may act as a good biomarker for visceral leishmaniasis [21].

Interestingly, the modest increase in CCL3 expression (0.21-fold) suggests selective modulation of chemokine pathways by L. infantum. Previous studies have shown that Leishmania species can manipulate chemokine signaling to evade immune detection. The differential regulation of chemokines observed in this study aligns with these findings, indicating a nuanced interplay between host and parasite [22].

The lack of gender-based differences in apoptosis rates and gene expression emphasizes the universality of these immunological responses. This is especially important for the development of diagnostic and therapeutic strategies, as it suggests that interventions targeting apoptotic and chemokine pathways would be effective across diverse patient populations [23].

From a clinical perspective, the results of this study have several implications. The increase in levels of Bcl2 and caspase-3 in lymphocytes and CCL4 and CCL8 in neutrophils establishes that these proteins serve as useful biomarkers for infection with Leishmania infantum. Moreover, this pathway may be beneficial in the creation of new drugs that might be used to treat visceral leishmaniasis. For example, Bcl2 inhibitors have been proved to induce apoptosis in cancer cells and thus may be used as anti-leishmania drugs. Similarly, modulating chemokine signaling could help restore immune homeostasis and mitigate disease progression [24].

5 Conclusion

In conclusion, this study underscores the intricate interactions between Leishmania infantum and host immune cells, shedding light on the molecular mechanisms underlying apoptosis and chemokine expression. The findings contribute to our understanding of visceral leishmaniasis pathogenesis and provide a foundation for future research aimed at developing targeted interventions. Further studies exploring the role of other chemokines and apoptotic regulators, as well as their interactions with parasite-derived factors, are warranted to fully elucidate the host-parasite dynamic.

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Table 1: Apoptosis and Necrosis Rates of Neutrophils and T Cells

Cell Type	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)
Neutrophils (treated)	37	29	28
Neutrophils (untreated)	11	8	4
T Cells (treated)	43	29	14
T Cells (untreated)	9	3	6

Table 2: Gene Expression Changes in Lymphocytes and Neutrophils

Gene	Fold Change (Lymphocytes)	Fold Change (Neutrophils)	p-value
Bax	-2.4	-	< 0.001
Bcl2	3.3	-	< 0.0001
Caspase-3	4.4	-	< 0.0001
CCL3	-	0.21	0.711
CCL4	-	1.14	0.0391
CCL8	-	3.38	< 0.0001

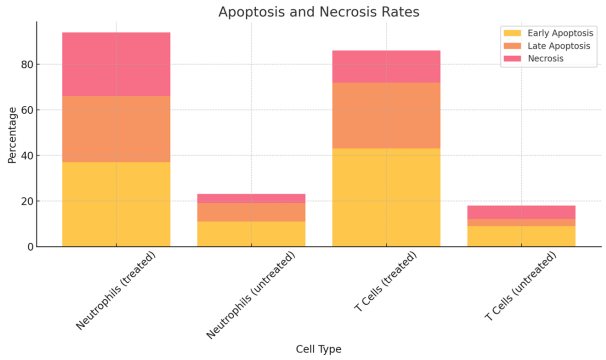

Figure 1: Apoptosis and Necrosis Rates in Neutrophils and T Cells.

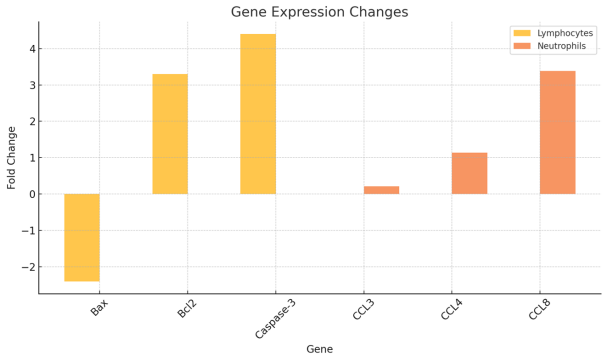

Figure 2: Gene Expression Changes in Lymphocytes and Neutrophils Exposed to *L. infantum*.