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Research Article

Apoptosis Induction and Expression of Apoptotic-Related and *CCL3, CCL4* and *CCL8* Genes in Lymphocytes and Neutrophils Exposed to the *Leishmania infantum* Parasite

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A B S T R A C T

Background: Neutrophils exposed to parasites produce chemokines that recruit diverse leukocytes, including T cells and dendritic cells, to the infection site.

Aim: This study aims to assess apoptosis induction and the expression of apoptotic-related genes (*CCL3, CCL4*, and *CCL8*) in lymphocytes and neutrophils exposed to the Leishmania infantum parasite.

Method: Blood samples were collected, and neutrophils and T cells were isolated and quantified. These cells were exposed to the infective stage of L. infantum and incubated in CO2 conditions for an hour. The apoptosis rate was assessed through flow cytometry. The expression of genes, including Bax, Acl2, caspase 3, CCL3, CCL4, and CCL8, was measured using quantitative real-time PCR (RT-qPCR), with the β-Actin gene employed as the reference.

Results: The mean apoptosis rates for neutrophils and macrophages exposed to promastigotes were 63% and 72%, respectively. In lymphocytes, the expression of the Bax gene decreased by 2.4 fold, while the Bcl2 and caspase 3 genes increased by 3.3 fold and 4.4 fold, respectively. The expression of the CCL3, CCL4, and CCL8 genes in neutrophils increased by 0.21 fold, 1.14 fold, and 3.38 fold, respectively.

Conclusion: Exposure to L. infantum significantly increased the apoptosis rate of neutrophils and macrophages, which led to a significant increase in the expression of Bcl2 and caspase 3 apoptotic genes, and a decrease in the expression of the Bcl2 anti-apoptotic gene in lymphocytes. Furthermore, the CCL4 and CCL8 chemokine genes in neutrophils increased significantly which can be diagnostic and therapeutic targets.

Keywords: Apoptosis, Lymphocytes, Neutrophils, Leishmania Infantum

Introduction

Leishmaniasis is a zoonotic protozoan infection which is transferred by mosquito vectors and infects nearly 20 million people worldwide.[1,2] Moreover, humans, dogs and rodents act as reservoirs. Leishmaniasis occurs in three forms, of which the visceral or systemic form is caused by *Leishmania infantum* (L. infantum).[3,4] The disease is distributed in the Eastern Mediterranean countries.[5,6] Chemokines are chemotactic cytokines produced by the immune cells such as neutrophils and participate in leukocyte trafficking through the recruitment of T cells, dendritic cells, natural killer (NK) cells, monocytes and neutrophils into the site of injury or inflammation.[7,8] CC and CXC-type chemokines are produced in various tissues by leukocytes, endothelial, epithelial and fibroblast cells. Neutrophils also participate in the primary stage of bacterial and fungal infections via the Neutrophil Extracellular Traps (NETs) mechanism. These cells also fight against *L. infantum* via an opsonin-independent pathway and produce several cytokines. Chemokines play fundamental roles in the activation of innate and adaptive immunity against cutaneous leishmaniasis.[8,9] Moreover, early exposure to Leishmania species causes the production of CXCL8 which establishes the neutrophil activation and response. The mosquito saliva contains CCL2 which has a chemotactic effect on macrophages. The CCL2, CXCL9 and CXCL10 in humans play an important role in combat against Leishmania spp.[8–10]

The human macrophages negatively regulate the CCR1 chemokine receptor which has a potential role in the macrophage function. The aim of our research included assessment of apoptosis induction and expression of apoptotic-related and *CCL3*, *CCL4* and *CCL8* genes in lymphocytes and neutrophils exposed to the *L. infantum* parasite.

Methods

A total of 100 participants, with age between 20 and 30 years, sequentially enrolled at Al-Sader Teaching Hospital in Al-Najaf between August 2023 and March 2024. The current study performed according to the rules issued by the research committee of the medical ethics unit under the Ministry of Health in Iraq, informed and written permission was obtained from each participant (ethical approve 127-24).

Sampling and Culture Medium

Blood sampling (10 mL) was performed on 100 healthy individuals aged 20–30 years (50 men and 50 women). The RPMI1640 (Gibco-us) medium was purchased and supplemented with 15% foetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). The *L. infantum* JPCM5 strain was purchased and cultured into the NNN medium and fresh culture of the parasite at the logarithmic phase was added to the RPMI medium.

Neutrophil and T Cell Isolation

Firstly, 7 mL of Ficoll was added into a 50 mL falcon and 10 mL of peripheral blood was added and centrifuged at 10000 rpm for 30 min at ambient temperature. Next, the inter-phase layer containing lymphocytes was obtained, washed using RPMI, counted and preserved. The viability of cells was confirmed using trypan blue dye. At the next stage, the cells were cultured into the RPMI medium and the suspension of promastigotes was added and incubated for one hour. Moreover, similar culture conditions were performed for the T cells.

Flow Cytometry

The flow cytometry method was employed for the assessment of the apoptosis and necrosis rate of neutrophils and T cells. For the identification of apoptotic and necrotic cells, the Annexin-V dye conjugated with fluorescein isothiocyanate (FITC, BD, USA) and propidium iodide (PI, BD, USA) were used. The cells were treated with 10^6 cells of parasite for 48 hours next, trypsin was added and cells were washed twice using phosphate-buffered saline (PBS) and centrifuged at 1500 rpm for 5 min. The binding buffer containing 5 µL of Annexin V conjugated with FITC and 5 µL of PI and kept at dark conditions for 15 min and subjected to a flow cytometry device (FACSCalibur, BD, USA) for analysis.

Gene Expression

The total RNA was extracted from neutrophils and T cells using a Mini Plud kit (A&A Biotechnology, Poland, cat number 036-100) as per the protocol of the manufacturer and kept at −80 °C. The RNA quality and quantity were evaluated using a NanoDrop spectrophotometer (ND200C; Fisher Scientific, Hampton, PA, USA). Next, the cDNA was synthesised from 1000 ng of each total RNA using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The cDNA samples were stored at -20 °C. The real-time PCR technique was performed according to the Jaworska method. The delta-delta CT method was employed for the gene expression. Single neutrophils or T cells were considered as the control population.[11]

Statistical Analysis

The SPSS version 20 was used and chi-square and ANOVA tests were applied with 0.05 significance level.

Results

Apoptosis of Neutrophils and T Cells

The mean apoptosis rates of neutrophils exposed to promastigotes were 63% ($p = 0.033$) and 72% ($p = 0.0011$), respectively (Table 1). There was a significant difference in

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early apoptosis of treated and untreated neutrophils ($p < 0.001$). In addition, significantly higher rates of early and late apoptosis of treated T cells were observed compared to untreated T cells ($p < 0.0001$, Table 1). Furthermore, a significantly higher rate of necrosis was observed for both treated neutrophils and T cells ($p < 0.0001$ and $p < 0.001$, respectively). Noticeably, there was no significant difference between both genders regarding neither the apoptosis nor necrosis rate.

Table 1.Apoptosis and Necrosis Rate of Cells Exposed to Promastigotes and Control Cells

Cells	Early Apoptosis	Late Apoptosis	Necrosis
Treated neutrophils (%)	37	29	28
Untreated neutrophils (%)	11	8	4
p value	< 0.001	< 0.001	< 0.0001
Treated T cells (%)	43	29	14
Untreated T cells (%)	9	3	6
p value	< 0.0001	< 0.0001	< 0.001

Gene Expression

The expression of Bax in lymphocytes was decreased 2.4 fold ($p < 0.001$) and those of Bcl2 and caspase 3 increased 3.3 ($p < 0.0001$) and 4.4 fold ($p < 0.0001$), respectively. Additionally, the expression of *CCL3*, *CCL4* and *CCL8* genes in neutrophils increased 0.21 fold ($p = 0.711$), 1.14 fold ($p = 0.0391$) and 3.38 fold ($p < 0.0001$), respectively (Figures 1 and 2).

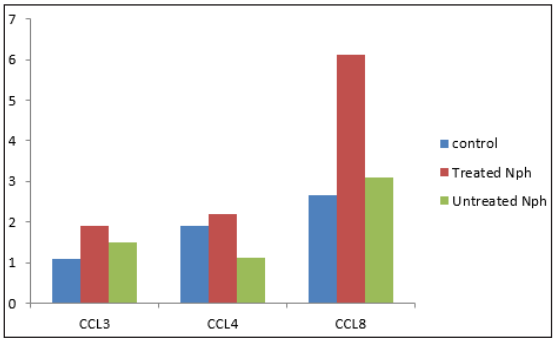

Figure 1.Expression of Chemokine Genes in Neutrophils (Nph), where the CCL8 had a Significant Difference in the Treated Group Compared to Untreated and Control Groups

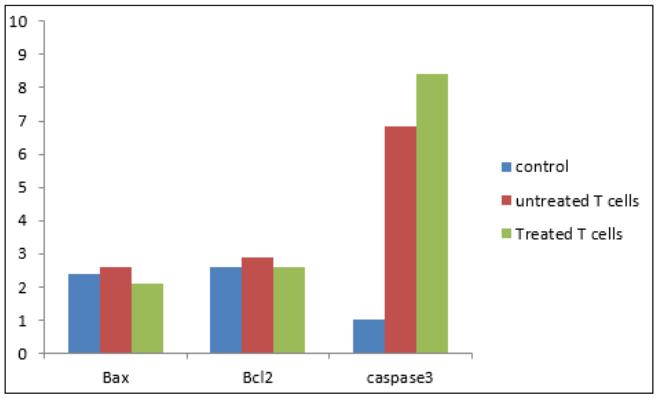

Figure 2.Expression of Apoptosis-Associated Genes, where the Bax Gene was Significantly Decreased and Bcl2 and caspase3 were Significantly Increased in Treated T Cells

Discussion

In this study, we observed a significant induction in the apoptosis rate of neutrophils and T cells exposed to promastigotes included. Moreover, there was a significant difference in early apoptosis of treated and untreated neutrophils ($p < 0.001$).

In a previous study, infection of gp91phox-/- mice with *Leishmania* amazonensis has been associated with neutrophil accumulation but not CD4+ T cells mediated immunity in the infection site. Moreover, the infection-mediated apoptosis was deficient and neutrophil necrosis was high.[12]

In addition, we observed significantly higher rates of early and late apoptosis of treated T cells compared to untreated T cells ($p < 0.0001$). Furthermore, a significantly higher rate of necrosis was observed for both treated neutrophils and T cells ($p < 0.0001$ and $p < 0.001$, respectively. Noticeably there was no significant difference between both genders regarding neither the apoptosis nor necrosis rate.

The exhaustion of T cells is a crucial factor in the progress of visceral *leishmania*sis. Both CD4+ and CD8+ cells participate in the control of L. donovani infection and several microRNAs have participated in T cells apoptosis during infection by L. donovani.[13]

In our study, the expression of Bax in lymphocytes decreased by 2.4-fold ($p < 0.001$) and those of Bcl2 and caspase 3 increased by 3.3 ($p < 0.0001$) and 4.4-fold ($p < 0.0001$), respectively.

Additionally, the expression of *CCL3*, *CCL4* and *CCL8* genes in neutrophils increased 0.21 fold ($p = 0.711$), 1.14 fold ($p = 0.0391$) and 3.38 fold ($p < 0.0001$), respectively (Figures 1 and 2). The expression of CCL4 and CCL8 genes contributes to the establishment of neutrophil potential responses and inhibition of parasite multiplication following infection of L. infantum. On the other hand, chemokines activate

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macrophages.[14–16] *Leishmania* donovani has also induced the expression of CCL5 and chemokines in macrophages. Furthermore, *Leishmania* major infection causes an increase in CCL7 secretion. However, the MIP-1α chemokine has played a dual role in the parasite infection. Th1 cells also play an important role in the control of *Leishmania* spp. infection.

Conclusion

The exposure to *L. infantum* significantly increased the apoptosis rate of neutrophils and macrophages. Notably, this exposure significantly increased the expression of Bcl2 and caspase 3 apoptotic genes and decreased the expression of the Bcl2 anti-apoptotic gene in lymphocytes. Furthermore, the CCL4 and *CCL8* chemokine genes in neutrophils increased significantly which can be as diagnostic and therapeutic targets.

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Conflict of Interest: None

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