Original Article

The Role of Lipopolysaccharide (LPS) in Modulating the Immune Reaction after Infected with *Leishmania Donovani* in Albino Mice

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Abstract

Aim: The aim of this study was to investigate the effect of LPS in immune response through detecting some immune markers by IHC technique in the liver of mice infected with *Leishmania donovani* (VL). **Methods:** Three groups of eighteen mice were created: the first was control negative (non-infected), the second was control positive (infected with 2X107 promastigotes of *Leishmania donovani),* and the third group was infected with 2X107 promastigotes of *Leishmania donovani* and treated with 40 ng/ml of LPS. The treatment was given orally twice daily for one month. Mice were dissected, and the liver separated for an immunohistochemistry study for the markers (BCL-2, Caspase-3, CD3, CTLA-4). **Results:** The results showed there was significant elevation ($p < 0.05$) in the expression intensity (EI) for the markers in liver tissue of the control positive (infected) group compared with the control negative group and the group of mice infected with LV and treated with LPS; the EI (%) according to Aperio image program analysis were (0.90, 0.97, 0.93, 0.90) in the control positive, (0.82, 0.83, 0.85, 0.85) in the control negative, and (0.81, 0.89, 0.87, 0.84) in the group of mice that were infected with LV and treated with LPS. **Conclusion:** The conclusion: 40 ng/ml of LPS had a role in providing protection to the liver from the effects of LV infection by modifying the innate and acquired immune response as a result of the decrease in the intensity of expression of BCL-2, Caspase-3, CD3 and CTLA-4, which also resulted from the occurrence of a reciprocal relationship between macrophages, hepatic parenchymal cells and non-parenchymal cells.

Keywords: BCL-2, Caspase-3, CD3, CTLA-4, *Leishmania Donovani*, LPS.

Introduction

A group of anthropozoonotic diseases known as leishmaniasis are caused by various trypanosomatid species of the *Leishmania* genus, each of which is responsible for distinct pathologies.[1] Medicinally, leishmaniasis can show up as visceral, mucocutaneous, or tegumentary (localised, disseminated, or diffuse). The most severe type of disease is called kala azar, or visceral leishmaniasis (VL). In sub-Saharan Africa and India, *L. donovani* can cause the disease, while in southern Europe, North Africa, and Brazil, *L. infantum* can. A severe type of L. infantum/donovani infection may develop even if 80–90% of those infected do not show any symptoms. where patients may progress to haemorrhagic symptoms, hepatosplenomegaly, persistent fever, severe anaemia and leukopenia, and sepsis.[2]

Gram-negative bacteria's outer membranes depend on lipopolysaccharides (LPS), large amphipathic glycoconjugates with a distal polysaccharide and a core oligosaccharide joined by a hydrophobic lipid domain. These proteins are also called lipoglycans because they contain lipids and sugar.[3] The majority of bacterial LPS molecules cause inflammation in mammals and are thermostable. LPS

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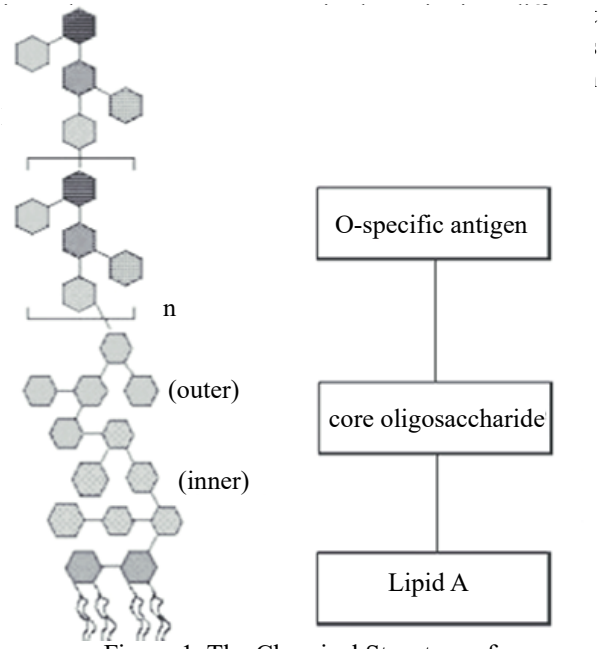

Figure 1: The Chemical Structure of Lipopolysaccharide. LPS Consists of an O Specific Antigen, a Core Oligo-saccharide and a Fatty Acid Tail Called Lipid A.s.

A member of a conserved protein family is caspase-3. Activated proteolytic roles in apoptosis execution are well recognised, and it is extensively expressed. Cells tend to respond to certain extrinsic or intrinsic inducers of this method of cell death. Nonetheless, mounting data indicates that in multicellular organisms, caspase-3 regulates the development and homeostatic upkeep of both healthy and cancerous cells and tissues.[6] All caspases are first generated as procaspases, which are zymogens that are dormant and may be triggered by a number of different internal and/or external stimuli.[7] According to certain experiments, caspase-3 plays a critical role in the death of cells in a unique cell-tissue type or death stimulus-specific pattern. It also plays a significant role in some biochemical alterations linked to the initiation and completion of apoptosis.[8] According to Kumar *et al.*[9], the activity of caspase 3 was significantly ($p < 0.001$) higher in animals infected with VL than in controls, and it was significantly ($p = 0.007$) higher in animals infected with the parasite at the 90th day.

There are two ways that apoptosis can occur: the intrinsic pathway and the extrinsic pathway.[10] The B cell lymphoma 2 (BCL2) protein family, which controls mitochondrial outer membrane permeabilisation (MOMP), is responsible for the intrinsic apoptotic pathway, also known as the mitochondrial pathway.[11] Based on their intracellular functions and structural characteristics, this protein family can be categorised into three groups. One group consists of BAK (BCL2 antagonist/killer) and apoptotic effectors, such as BAX (BCL2-associated X protein).[12] The second group, referred to as pro-survival or anti-death BCL2 family members, suppresses death by binding and sequestering their pro-death counterparts. Only BH3 proteins make up the last category. These proteins share a 15–25 residue BH3 domain with other members of the BCL2 family. However, the BH3 domain is required to control MOMP and interact with other members of the BCL2 family.[13] Both in vitro and in vivo experimental setups were used by Pandey *et al.*[14]. They demonstrate that the Bcl-2 protein is expressed more when infected with L. donovani. They further demonstrate that when Bcl-2 is reduced or functionally inhibited, the parasites are eliminated more rapidly. Their research shows that by inhibiting NO generation via Bcl-2 activation, the cytokine IL-13 has a pro-parasitic effect. They further show that the host surface toll-like receptor (TLR)-2 is involved in the initial contact and subsequent internalisation of the parasites by the macrophages, as well as in the expression of Bcl-2. Bcl-2 small molecule inhibitors may now be employed as antileishmanial medications, according to this material.

The mechanism by which Leishmania aethiopica and Leishmania mexicana spread was examined by Ranatunga *et al.*[15]; they found that active caspase-3 expression was found to significantly increase during L. aethiopica spreading but not during L. mexicana spreading ($P < 0.05$). TCR cell surface expression and signal transduction require the cell surface complex CD3, which has four subunits. T cell antigen receptor (TCR) binding integral membrane glycoprotein chains make up these subunits.[16] At the cell surface, CD3 is co-expressed with the TCR complex and is expressed as either TCR alpha/beta or TCR gamma/delta heterodimers. When T cells develop, CD3 is initially expressed in their cytoplasm before moving to the membrane as they mature.[17] It offers the initial signal that the lymphocyte's TCR detects to initiate its activation. As a result, it turns into a highly desirable receptor to control the immune response in various situations, from tolerance induction to immune activation.[18] The CD3 antigen is a useful immunohistochemical marker for T cells in tissue sections and is an excellent T cell marker for detecting both normal T cells and T cell neoplasms (lymphomas and leukaemias) due to its specificity for T cells and appearance at all stages of T cell development.[19] According to the results of a study conducted on patients with visceral leishmaniasis, the percentage of CD3 decreased, but the percentage of CD3 significantly increased in treated patients.[20] According to another study, the expression of CD3 cells in the liver and spleen of dogs infected with VL did not have a cytotoxic effect on parasitised macrophages. The low percentage of CD3+ phenotypes in the infected group indicates that, regardless of the parasite load in each organ, this cytotoxic method does not appear to be effectively activated in dogs with VL.[21]

The surface of activated T cells contains the immune checkpoint receptor known as cytotoxic T-lymphocyte antigen 4 (CTLA-4). CTLA-4 functions as one of the immune system's "off" switches, reducing or stopping an immune response. T cell activation is suppressed when CTLA-4 attaches to its ligand on antigen-presenting cells

(APCs), maintaining equilibrium when the immune system is overactive.[22]

Research conducted on animal models demonstrated that CTLA-4 inhibits the immune responses against Leishmania by triggering the anti-inflammatory cytokine transforming growth factor-β (TGF-β).[23] Murray *et al*.[24] found that when anti-CTLA-4 antibody was administered to viscerally infected animals with L. donovani, the immune cells killed the parasite. The aim of this study was to investigate the effect of LPS in immune response through detecting some immune markers by IHC in the liver of mice infected with VL.

Material and Methods

Culture of Parasite

Baghdad University College of Science graciously supplied the Leishmania donovani strain (DUAA/IQ/2005/MRU15), cultured at 25–26°C in Novy-MacNeal-Nicolle medium (NNN medium) enriched with 100 IU/ml gentamycin.[25]

Preparation of LPS Concentration

Ten (10) mg of LPS (Medchemexpress, HY-D1056) were dissolved in 100 ml of PBS (phosphate buffer saline) in order to make the stock solution:

10 mg / 100 PBS = 0.1 mg (stock solution), and then 40 ng/ml of LPS was prepared by using the below formula, and then they were stored until use[26]:

C1 V1 = C2 V2

C1 = 0.1 mg (100000 ng) (1 mg = 1000000 ng)

V1 = 100 ml PBS

C2 = 40 ng/ml

V2 =?

In Vivo Study

At the Cancer Research Centre/Al-Mustansiriya University, we acquired 18 male Balb/c mice. At 12 weeks of age, each mouse weighed 20-25 grammes and was in good health. All mice were then divided into three groups, each one consisting of 6 mice as follows:

Uninfected group (negative control group).

2. The infected and untreated group (positive control group) were inoculated intraperitoneally with 2X107 promastigotes of Leishmania donovani (VL).

3- Infected group with 2X107 promastigotes of (VL), then treated with LPS (40) ng/ml.

The treatment was given orally twice daily for one month. After the end of the treatment period, the mice were anaesthetised using gas A (to exclude any harm that the mice may feel if the dissection is done without anaesthesia). Mice were dissected, and the liver was separated for immunohistochemistry study.

Behairy *et al*.[27] method was used to stain histological sections of the liver using the IHC technique. After loading the liver tissue sections onto positively charged glass slides, they were passed through xylene for dewaxing and then passed through a descending series of ethyl alcohol. Peroxidase blocking solution (100 **µl**) was added to the sections to deplete natural peroxidase. 50 **µl** of bovine serum albumin was added to each section, then 100 **µl** of primary antibodies (anti-BCL2, anti-Caspase-3, anti-CD3, and anti-CTLA4) were added. Biotinylated secondary antibodies (100 **µl**) were added; also, 100 **µl** of HRP and 200 **µl** of substrate working solution were added to each section. The sections were stained with haematoxylin, then passed through an ascending series of ethyl alcohol. It is necessary to wash the slides with PBS solution after each step, then the slides were loaded with DBX. The images of sections were analysed by the Aperio ImageScope program to evaluate the expression intensity.

Statistical Analysis

SAS was used to analyse data.[28] And used the Duncan test polynomial to compare significant average differences.[29]

Results

Control Negative Group (uninfected)

The results of microscopic examination of liver sections in the negative control group (Figure. 2) showed mild expression intensity of BCL-2in the cytoplasm of hepatocytes, while it was low in the plasma membrane. Based on Aperio Image System analysis, the expression intensity of BCL-2 was weak, and the percentage of expression in positive areas was 0.82%. The results of Caspase-3 expression in liver sections of the negative control group showed a mild expression intensity in the nucleus of hepatocytes and weak in the cytoplasm of hepatocytes. Based on Aperio Image System analysis, the percentage of expression intensity in the positive areas reached 0.83%. As for the marker CD3, its expression intensity was moderate in the sections of liver in negative control animals, and its expression intensity in positive areas reached 0.85% based on Aperio Image System analysis. Figure 2 showed the expression intensity of CTLA4, which was low in hepatic tissues and reached 0.85% in the positive areas (Table 1).

Control Positive Group Infected with Leishmania Donovani Strain (VL)

IHC results of liver sections in animals infected with the VL showed a high intensity of expression of BCL2 in the plasma membrane of hepatocytes and a low intensity in the hepatic sinusoids (Figure 3). Aperio Image System analysis showed that the intensity in the positive areas reached 0.90%. The results of Caspase-3 showed that the expression intensity was high in the cytoplasm of hepatocytes and moderate in other areas (plasma membrane, liver sinusoids), as in Figure 3, the percentage of expression intensity in the positive areas reached 0.97%. The result showed the moderate expression intensity of CD3 in the plasma membrane and hepatic sinusoids and little in the cytoplasm of hepatocytes in animals infected with VL, and the percentage of expression in the positive areas based on Aperio Image System analysis was 0.93%. The intensity of CTLA4 expression in the liver of infected animals was moderate in the plasma membrane and low in the cytoplasm of hepatic cells (Figure 3), and the intensity of expression in the positive areas reached 0.90% (Table 1).

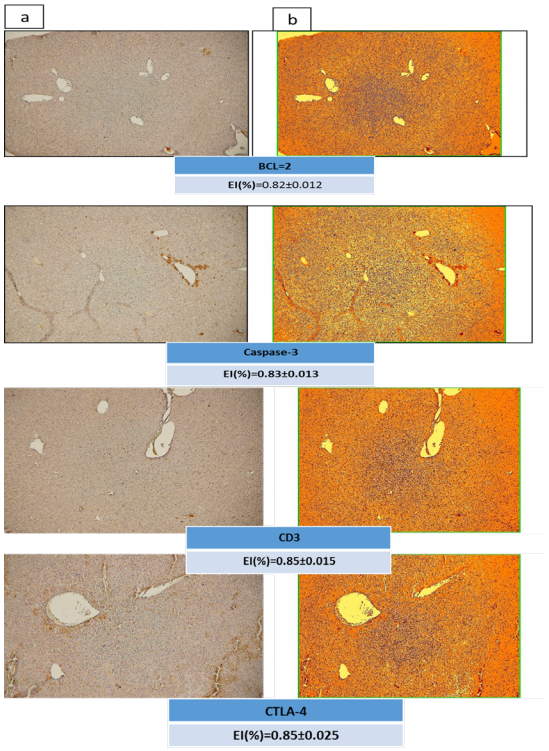

Figure 2: Cross Sections in Liver Control Negative Mice Showed the Expression of BCL-2, Caspase-3, CD3 and CTLA-4. A: All Sections Stained by IHC Technique (10X), b: Aperio Image Analysis Showed the Expression Intensity EI (%) =Mean±S. E.

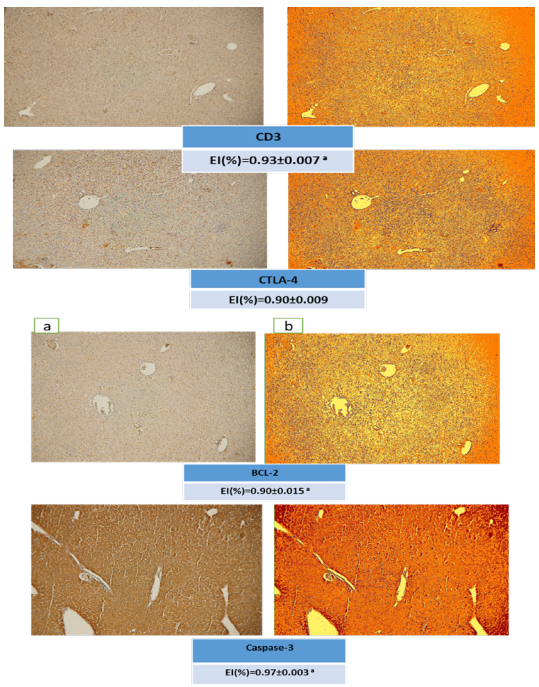

Figure 3: Cross Sections in Liver Control Positive Mice (infected by LV) showed the Expression of BCL-2, Caspase-3, CD3 and CTLA-4. A: All Sections Stained by IHC Technique (10X), b: Aperio Image Analysis Showed the Expression Intensity EI (%) =Mean±S. E.
[a] significant difference vs. control (-ve)

Infected mice with Leishmania Donovani Strain (VL) and Treated by LPS:

The results of BCL-2 in liver sections of animals infected with VL and treated with LPS showed the presence of low expression intensity in the cytoplasm and plasma membrane of hepatocytes (Figure 4), and the percentage of expression intensity in the positive areas is 0.81%, based on the analysis of the Aperio Image System program. Figure 4 shows the intensity of caspase-3 expression in liver sections of animals infected with VL treated with LPS; it was moderate in the cytoplasm, nucleus, and liver sinusoids, while the results of the Aperio Image System analysis indicated the presence of strong expression intensity in the positive areas, especially in the liver sinusoids and the portal area, and the percentage of intensity was 0.89% (Table 1). It was also observed that there was a moderate intensity of expression of CD3 in the plasma membrane, mild in the cytoplasm of hepatocytes, and low in the sinusoids in liver sections of mice infected with VL and treated with LPS (Figure 4). As for the result of the digital analysis, which showed the presence of a positive intensity in the plasma membrane and in blood vessels. Its percentage was 0.87% (Table 1). The results of CTLA4 in the liver sections of this group showed that there was little expression intensity in the plasma membrane and moderate expression in the cytoplasm of hepatic cells and blood vessels (Figure 4). The percentage of expression intensity in the positive areas was 0.84% based on Aperio Image System analysis (Table 1).

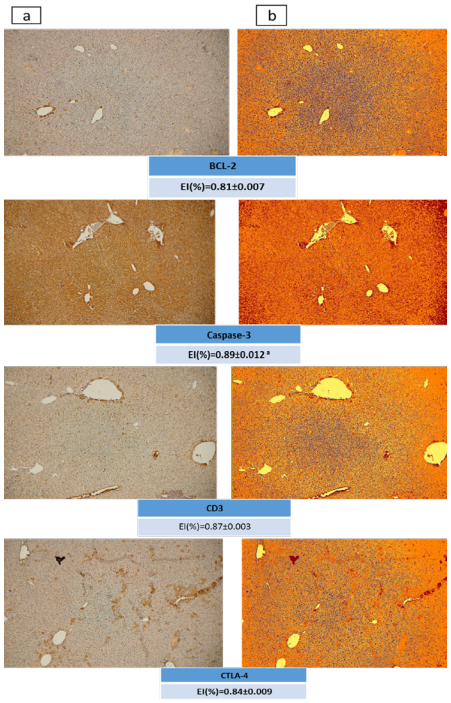

Figure 4: Cross Sections in Liver Infected Mice by LVand Treated by LPS (40ng/ml) Showed the Expression of BCL-2, Caspase-3, CD3 and CTLA-4. A: All Sections Stained by IHC Technique (10X), b: Aperio Image Analysis Showed the Expression Intensity EI (%) =Mean±S. E.

The results of the statistical analysis (Table 1) showed that there was a significant increase ($p<0.05$) in the expression intensity of the BCL-2 marker in the liver tissue of mice infected with VL compared to the negative control group and the group infected and treated with LPS (40 ng/ml); there was a significant elevation ($p<0.05$) in the expression intensity of Caspase-3 in the positive control group (infected with VL and treated with LPS (40 ng/ml)) compared with the negative control group. There was also a significant increase ($p<0.05$) in the percentage intensity of CD3 expression in the positive control group (infected) compared with the negative control and the group of mice infected with VL and treated with LPS, but the intensity was non-significant between the negative control and the group infected and treated, while there was a significant elevation ($p<0.05$) in the percentage intensity of CTLA4 expression in the group of animals infected with VL compared with the expression intensity in the group infected with VL and treated with LPS.

There was a significant correlation ($p < 0.05$) between BCL-2 and CD3 ($r = 0.73$), CTLA4 and CD3 ($r = 0.80$), BCL-2 and caspase-3 ($r = 0.74$), and CD3 and caspase-3 ($r = 0.89$) (Table 2).

Table 1: Showed the Significant Differences in Expression Intensity (EI) for BCL-2, Caspase-3, CD3 and CTLA-4 between the Groups of the Study (control negative (-ve), Control Positive(+ve) Infected by LV and Group that Infected by LV and Treated by LPS.

Parameter	Groups	EI (%) (Mean±S.E)	P value
Bcl-2	Control (-ve)	0.82±0.012	0.003**
	Control (+ve) (Infected)	0.90±0.015 [a]	0.001**
	Infected+Treated	0.81±0.007	
Caspase-3	Control (-ve)	0.83±0.013	0.005**
	Control (+ve) (Infected)	0.97±0.003 [a]	0.001**
	Infected+Treated	0.89±0.012 [a]	
CD3	Control (-ve)	0.85±0.015	0.002**
	Control (+ve) (Infected)	0.93±0.007 [a]	0.004**
	Infected+Treated	0.87±0.003	
CTLA-4	Control (-ve)	0.85±0.025	0.06 NS
	Control (+ve) (Infected)	0.90±0.009	0.03*
	Infected+Treated	0.84±0.009	

* Significant differences, NS= Non- significant No, [a]significant difference vs. control (-ve)

Table 2: Showed the Significant Correlation ($p<0.05$) of Expression Intensity (EI) between the Parameters (BCL-2, Caspase-3, CD3, CTLA-4) among the Groups of the Study.

Parameter		CTLA-4	CD3	Caspase-3	Bcl-2
CTLA-4	Pearson Correlation (r)	1			
	Sig. (2-tailed)				
CD3	Pearson Correlation (r)	0.806**	1		
	Sig. (2-tailed)	0.009			
Caspase-3	Pearson Correlation (r)	0.693*	0.898**	1	
	Sig. (2-tailed)	0.038	0.001		
Bcl-2	Pearson Correlation (r)	0.604	0.734*	0.745*	1
	Sig. (2-tailed)	0.085	0.024	0.021	

Discussion

The results of the current study showed a significant increase in the intensity of BCL-2 expression in the plasma membrane of hepatocytes in the liver tissue of the group of mice infected with VL compared to the negative control group and the group infected with VL and treated with LPS.

BCL-2 is an anti-apoptotic mitochondrial protein. Some parasites mutate infected cells into cancer-like cells so that they can evade the immune response. Some studies have indicated an increase in the level of proteins regulating the cell cycle, including BCL-2, and a decrease in the expression of proteins responsible for autophagy and apoptosis as a result of infection with the Plasmodium parasite.[30]

Results of the current study agreed with the result of[14] in terms of the increase in BCL-2 expression in cells infected with the *Leishmania donovani* parasite, as BCL-2 works to inhibit the enzyme Nitric Oxide Synthase (iNOS) and thus prevent the formation of Nitric Oxide, which is one of the toxic and deadly substances for the parasite. Reversing this condition by inhibiting BCL-2 expression or using anti-BCL-2 stimulates the enzyme (iNOS) to form nitric oxide, which leads to killing the parasite and stimulating the mechanisms of programmed death in infected cells.

This is consistent with the results of the current study, as there was a decrease in the intensity of BCL-2 expression in the liver tissue of the group of mice infected with LV and treated with LPS (40 ng/ml), indicating the role of LPS at this concentration in affecting BCL-2 expression and stimulating cell death mechanisms or stimulating infected cells to produce toxic agents that eliminate the parasite and then heal the liver tissue from infection.

The results of the current study showed a significant

increase in the intensity of caspase-3 expression in the cytoplasm of hepatocytes in the group of mice infected with VL compared to mice in the negative control group and the group infected and treated with LPS. The mechanism used by L strains of parasites is to stimulate host cells to release TNF-alpha and GM-CSF and inhibit the release of IL-1 beta and M-CSF by infected macrophages, thus inhibiting their entry into apoptosis.[31] Study by Bosurgi and Rothlin[32] indicated that protozoa induce programmed death in uninfected parenchymal cells and non-parenchymal cells (NPC) that are responsible for the hepatic immune response, which leads to the parasite escaping the immune response and continuing its survival and spread. This explains the high expression of caspase-3 in the cytoplasm and plasma membrane of hepatocytes in the liver tissue of animals infected with LV in this study, which is likely to be in uninfected parenchymal cells or in NPC cells.

The decreased expression of caspase-3 in the liver tissue of the group of mice infected with LV and treated with LPS is likely due to the role of LPS in inhibiting the mechanisms of the L. donovani parasite that ensure its survival and continued infection. Some studies have indicated that low concentrations of LPS stimulate M2 macrophages in liver tissue, which play a role in protecting the liver from any harmful factors it may be exposed to. Also, low doses of LPS do not induce the release of an appropriate amount of inflammatory cytokines necessary for cellular damage to occur in liver tissue.

In this study, CD3 expression intensity was increased in the liver tissue of mice infected with LV compared to its decreased expression in the negative control group and the group infected with LPS. The CD3 receptor is associated with the TCR receptor, which is expressed by all types of T cells, whether CD4 or CD8.[18]

The increase in CD3 expression in liver tissue is a result of the infiltration of various T cells, including CD3CD8 and CD3CD4, as a result of the presence of LV infection.[27] CTLA-4 is an inhibitory checkpoint that binds to CD80/CD86 to stop the activity of T cells and is responsible for T-cell exhaustion. Yang *et al*.[33] indicated that CTLA-4 induces the inhibition of antiparasitic immune activity, as it inhibits the release of inflammatory cytokines from cytotoxic T cells and at the same time automatically stimulates the formation of regulatory T cells and the release of anti-inflammatory mediators such as IL-10 and TGF-β.[34] The role of CTLA-4 is important in inducing CD4 tolerance *in vivo* in the liver, which is synergistic with IL-10, which has a regulatory role in transmitting cellular signals to immune checkpoints, thus affecting the hepatic immune response.[35] In the current study, there was an increase in the intensity of CTLA-4 expression in the liver tissue of the group infected with LV, which induces a reduction in the inflammatory response, which is one of the escape mechanisms used by the parasite to bypass the immune response and protect infected cells, while treatment with LPS (40 ng/ml) after LV infection reduced the intensity of CTLA-4 expression in the liver tissue of this group. In a study conducted by Yang *et al*.[33], they noted that the use of anti-CTLA-4 led to the activation of CD4 and CD8 cells, especially Th1 and Th17 cells in the liver tissue, reversing the state of T cell exhaustion at the expense of Treg cells.

The increase in the intensity of CTLA-4 expression was consistent with the increase in CD3 expression in the group of mice infected with LV, indicating the presence of Treg cells in the liver tissue, which are responsible for inhibiting the immune role of other types of T cells. The decrease in the intensity of CTLA-4 expression was consistent with the decrease in the intensity of CD3 expression in the liver tissue of the group of mice infected with LV and treated with LPS, indicating its role in modifying the immune response against the parasite and limiting its spread.

The results of the statistical analysis showed a significant correlation between the intensity of CTLA-4 and CD3 expressions as well as with Caspase-3 in the experimental groups under study, indicating the possibility that the LV parasite modifies the innate and acquired immune response. The decreased expression intensity of the proteins under study in the group of mice infected with LV and treated with LPS (40 ng/ml) illustrates the role of LPS in providing protection to hepatic cells from the behaviour of the *L. donovani* parasite, which uses multiple defence mechanisms that enhance its survival. The effect of LPS depends on the concentration of the dose, as it has a biphasic function, meaning that it can be toxic in high doses and safe and modifies the immune response in low doses, as the low dose stimulates the restoration of the immune response and provides cellular protection for the liver tissue resulting from a reciprocal relationship between macrophages and the mechanisms of cell death, whether in the case of homeostasis or diseases.[36]

Conclusion

40 ng/ml of LPS had a role in providing protection to the liver from the effects of LV infection by modifying the innate and acquired immune response as a result of the decrease in the intensity of expression of BCL-2, Caspase-3, CD3 and CTLA-4, which also resulted from the occurrence of a reciprocal relationship between macrophages, hepatic parenchymal cells and non-parenchymal cells. The results of this study also showed a significant correlation between the proteins under study in the case of high or low intensity of expression of these proteins. The use of low-dose LPS could be a proposal for a future treatment project based on modifying the immune response to treat parasitic infections, especially LV infection.

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Conflict of Interests

None declared.

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