OX40+ T lymphocytes and IFN-γ are associated with American tegumentary leishmaniasis pathogenesis *

Linfócitos T OX40+ e IFN-γ estão associados com a patogênese da leishmaniose tegumentar americana

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Abstract: BACKGROUND: Leishmaniases are zoonoses considered a public health problem, representing a complex group of diseases with a broad clinical spectrum and epidemiological diversity. Leishmaniasis is caused by several species of protozoa of the genus *Leishmania*. The evolution of the pathology and the resolution of the leishmaniasis are dependent mainly on the *Leishmania* species involved, although the cytokine profile plays an important role in the development of the immune response.
OBJECTIVES: The purpose of our study was to evaluate the immune response of patients affected by lesions of cutaneous leishmaniasis by immunostaining of the OX40, CD20, IFN-γ and IL-4 proteins.
METHODS: The tissue samples were collected from indolent skin ulcers confirmed as cutaneous leishmaniasis of 41 patients aged between six and 90 years. The lesions were submitted to OX40, CD20, INF-γ and IL-4 immunolabeling.
RESULTS: We observed a statistically significant higher expression of IFN-γ compared with IL-4 (p=0.009). Besides, OX40 had higher expression when compared with CD20 (p<0.001).
CONCLUSION: The present study indicates that the immune response in lesions of cutaneous leishmaniasis is associated with a healing process, which can be explained by the higher expression of IFN-γ when compared with IL-4 protein levels.
Keywords: Cytokines; Immunohistochemistry; Leishmaniasis; Leishmaniasis cutaneous

Resumo: FUNDAMENTOS: As leishmanioses são zoonoses consideradas um problema de saúde pública, representando um grupo de doenças complexas, com uma diversidade de amplo espectro clínico e epidemiológico. A leishmaniose é uma doença causada por várias espécies de protozoários do gênero Leishmania spp. A evolução da patologia e a resolução da leishmaniose são dependentes principalmente da espécie de Leishmania envolvida; embora o perfil das citocinas tenha um importante papel no desenvolvimento da resposta imune.
OBJETIVOS: Proporcionar mais conhecimentos sobre os eventos inflamatórios na leishmaniose tegumentar através da avaliação da imunoexpressão de OX40, CD20, IFN-γ e IL-4.
MÉTODOS: Foram coletadas amostras de tecido de 41 pacientes, com idade variando entre 6 a 90 anos, com úlceras indolentes na pele confirmados através de exames de diagnóstico como leishmaniose tegumentar. As lesões foram submetidas a imunomarcação das proteínas OX40, CD20, IFN-γ e IL-4.
RESULTADOS: Observamos uma maior expressão de IFN-γ em comparação com IL-4, com diferenças estatisticamente significativa (p = 0,009). Além disso, OX40 tinha maior expressão quando comparada com IL-4 (p <0,001).
CONCLUSÃO: O presente estudo indica que a resposta imune nas lesões de LTA está associada a um processo de cura, que pode ser explicado pela maior expressão de IFN-γ quando comparadas com os níveis de IL-4.
Palavras-chave: Citocinas; Imunoistoquímica; Leishmaniose; Leishmaniose cutânea

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INTRODUCTION

The leishmaniases are zoonoses considered a public health problem, representing a complex disease with a broad clinical spectrum and epidemiological diversity.[1] In trying to understand the development of the immune response against Leishmania, rats and mice have been used in experimental models.[2] Unlike studies in animal models, studies of American tegumentary leishmaniasis (ATL) in humans demonstrated that the immune response is not well defined, however, they confirm the important role of the cytokine profile.[3] Depending on the immune response, focusing on the type of T helper cells involved in the infection, *Leishmania* can be characterized as resistant or susceptible, thereby promoting the expansion of subpopulations of Th1 and Th2 cells, respectively.[4] In disease resistance, there is a synthesis of cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α and susceptibility to infection is associated with the production of interleukin (IL)-4 and IL-10.[4] Recently, some of these studies investigated the ATL pathogenesis in humans, however the immune response remains to be elucidated in human infection. [5,6]

Indeed, although these studies described biological mechanisms associated with ATL pathogenesis, few studies yet dealt thoroughly with ATL lesions in humans. The purpose of our study was to evaluate the immune response in lesions of patients affected by ATL by immunostaining of the OX40 protein, preferentially expressed in CD4+ T cells, protein CD20, expressed in lymphocytes B, and cytokines involved in the immune response against *Leishmania*, IFN-γ and IL-4.

MATERIAL AND METHODS

In this study, we gathered 41 paraffin blocks taken from skin lesions of patients with confirmed diagnosis of ATL. These samples were from the north of Minas Gerais state, Brazil. The diagnosis of ATL was confirmed through biopsy, direct parasitological examination and/or Montenegro reaction. The cases were of primary manifestations with localized cutaneous clinical forms. The biopsies were performed on characteristic ulcers in the skin: seventeen on the lower limbs, nine upper limbs, one lip, one mouth, two face, one mandible, one nasal, two abdomen, one dorsal region and six at non-specified sites. These samples were collected from 41 patients ranging in age between six and 90 years. Of them, nine (21,95%) were female and thirty two (78,05%) male. The predominant histopathological pattern, obtained from the biopsies, was compound by hyperkeratosis, acantosis, eosinophils, giant cells and eventual granulomas. All patients were treated with pentavalent antimonial and they were completely clinically healed. Importantly, no patient had a recurrence.

Immunostaining was performed using 3μm sections of paraffin-embedded CL samples, fixed in 10% buffered formalin. All reactions followed standard protocols. Sections were deparaffinized and submitted to 10% ammonia hydroxide in 95% ethanol for ten minutes. Antigen retrieval was obtained by 10mM citric acid digestion, pH 6.0, using a steam cooker for the antibody IL-4, and 10mM Tris-EDTA digestion, pH 9.0, using a steam cooker for the antibodies OX40, CD20 and IFN-γ. After that, the slides were transferred to 10V H202, twice for 15 minutes and incubated overnight with primary antibody OX40 (dilution 1:100, BER-Act35, Santa Cruz, CA, USA), CD20 (1:100, O.N.85, Santa Cruz, CA, USA), IFN-γ (1:100, H145, Santa Cruz, CA, USA) and IL-4 (1:50, 8D4-8, Santa Cruz, CA, USA) followed by LSAB-HRP (Dako, Carpenteria, CA, USA). Reactions were developed with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, USA) containing and counterstained with hematoxylin. Negative and positive controls were also performed. As positive control, lymph nodes were used for all antibodies. Negative controls were obtained from samples without primary antibody staining but submitted to secondary antibody, DAB and counterstained with hematoxylin.

The immunohistochemical expression of biomarkers was evaluated using an Olympus® BH2 microscope (10× ocular and 40× objective lenses), and an ocular lattice (area 0.092 mm2) with 100 points composed by 10 horizontal and 10 vertical test lines superimposed on the test field to be measured. A total area of 1.84 mm2 was evaluated for each sample. Immunohistochemical analyses of all antigens investigated were performed by determining the percentage of positively stained cells in all fields counted on periepithelial areas and corium (10 fields for each specimen). This study was approved by Human Research Ethics Committee of the State University of Montes Claros.

STATISTICAL ANALYSIS

The data were analyzed by the Mann-Whitney test. All statistical analyses were performed with SPSS® (SPSS Inc, Chicago, IL, USA), version 18.0 for Windows®. P values < 0.05 were considered significant.

RESULTS

The immunohistochemical pattern of OX40, CD20, IFN-γ and IL-4 protein expressions and their association with some clinical parameters was demonstrated on figure 1. Analyzing the mean percentage of expression of positive immunostained cells, we noticed higher expression of OX40 when compa-

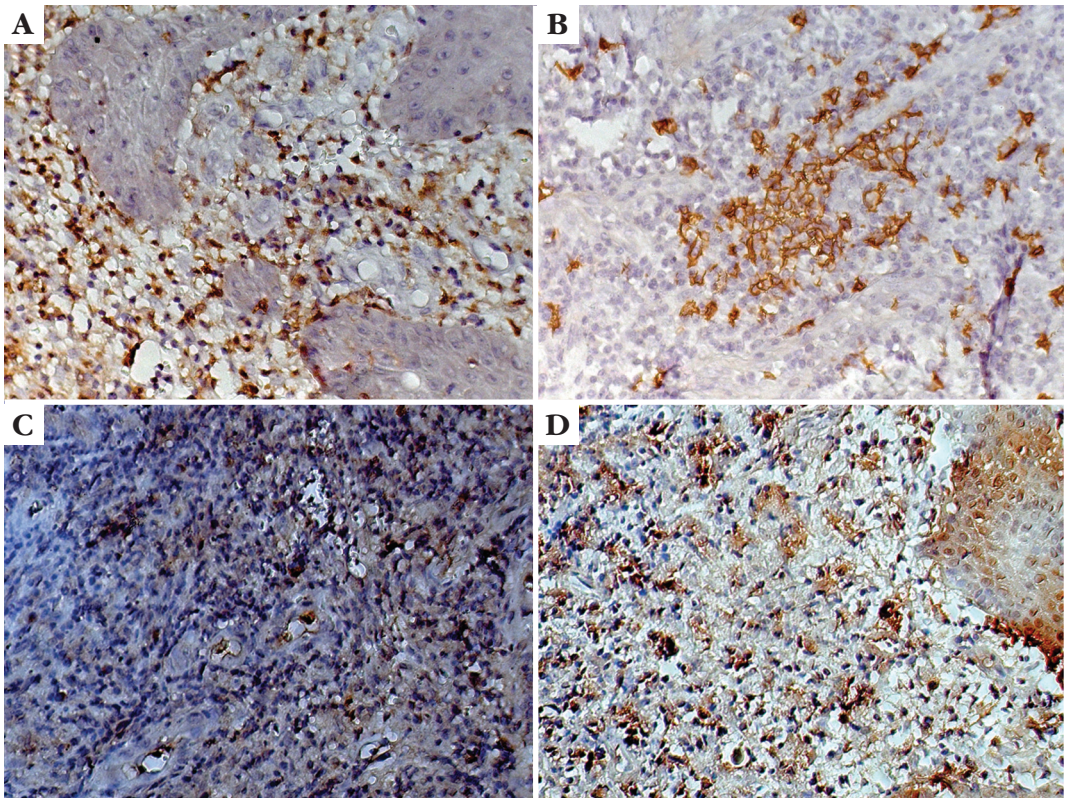


Figure 1: Immunostaining of OX40 (A), CD20 (B), IFN-γ (C) and IL-4 (D) on cutaneous leishmaniasis samples. LSAB/DAB. Original magnification of 400X

red to CD20 ($p < 0.001$). Moreover, to determinate the influence of cytokines on the regulation of the immune response, we compared the expression of cytokines IFN-γ and IL-4. We observed a statistically significant higher expression of IFN-γ when compared with IL-4 ($p = 0.009$). Besides, OX40 had higher expression when compared with IL-4 ($p<0.001$) (Graph 1).

DISCUSSION

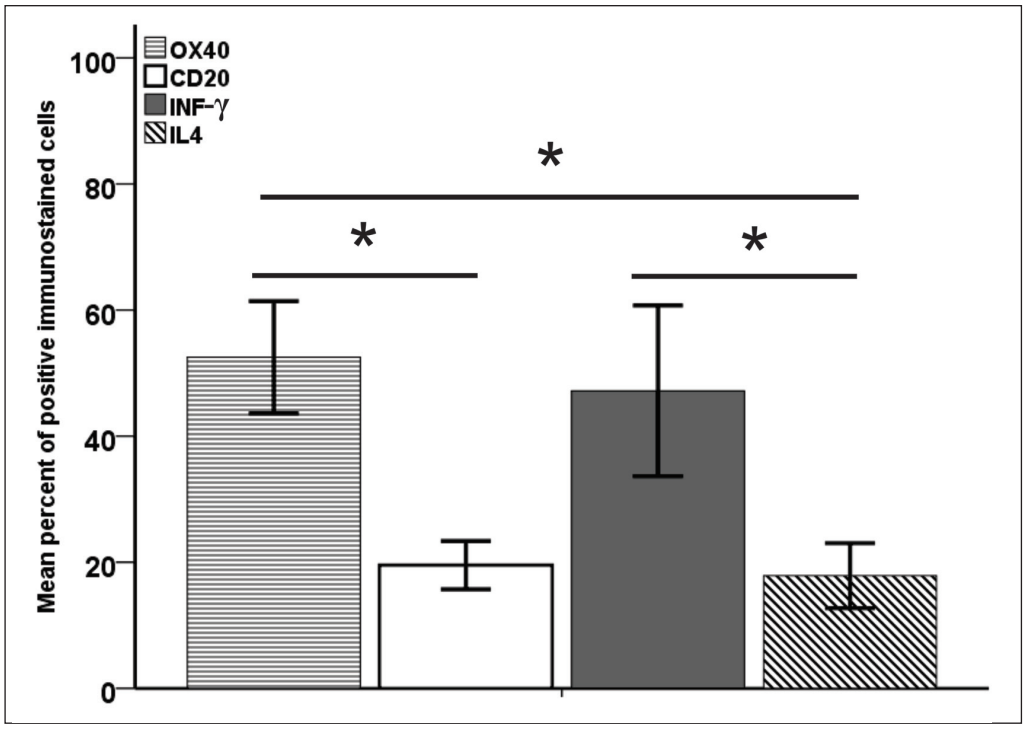
Leishmania infections are responsible for inducing the activation of a specific immune response in the host. This immune response is characterized by an expansion of various types of cells, mainly lymphocyte CD4 + T cells, characterized by a profile of Th1 and Th2 responses.[7]

OX40-OX40L pathway has been widely studied in relation to the health-disease process. It has been observed that OX40 signaling pathway has an important antiviral role and in autoimmune responses, and is also related to cancer. [8,9] However, few studies have dealt with the effects of the interaction OX40/OX40L on the differentiation of T helper cells (TH). Studies demonstrate that OX40 molecule appears to be related to a Th2 response, whereas in vivo studies have shown that this interaction is involved in both Th1 and Th2 responses.[10]

CD20 is a 33-37 kDa protein with four transmembrane domains and a small extracellular domain.[11] It is a non-glycosylated protein involved in the regulation of the activation, proliferation and differentiation of B lymphocyte cells.[12,13] Its expression is restricted to B lymphocytes, from the stage of pre-B cells until the late stage of differentiation, which, however, is not found in the plasma.[14]

Studies have reported that some cytokines such as interleukin 4 (IL-4), colony-stimulating factor and granulocyte macrophage, tumor necrosis factor α (TNF-α) and interferon α (IFN-α) are able to induce the expression of surface CD20.[15-17] However, the mechanisms regulating the expression of this protein are not well understood.[18] By analyzing the expression of the OX40 and CD20 proteins in the lesions of ATL, we observed higher expression, statistically significant, of the OX40 protein when compared to the CD20 protein. CD4 + T cells play an important role in coordinating the host immune response against infectious agents. These cells help phagocytic macrophages and dendritic cells to eliminate intracellular pathogens. In addition, CD4 + T cells also help B-lymphocytes in their response to antigen.[19]

In the present study, we observed higher expression of IFN-γ when compared with IL-4. IFN-γ, the main Th1 cytokine, has an essential role in con-



Graph 1: Average expression of OX40, CD20, IFN-γ and IL-4 in cutaneous leishmaniasis samples. Analyses by Mann-Whitney test showed a statistically significant difference between OX40 and CD20 ($p<0.001$), IFN-γ and IL-4 ($p=0.009$) and OX40 and IL-4 ($p<0.001$)

trolling infection against the *Leishmania*, through the activation of macrophages that are responsible for eliminating the parasites.[20,21] The IL-4 cytokine is associated with induction of Th2 immune response, thus inhibiting the activation of macrophages and the consequent escape of the parasite.[22] Studies have shown that IL-4 plays an important role in regulating the immune response, since this cytokine inhibits Th1 cell proliferation and hence there is a reduced synthesis of IFN-γ, which leads to inactivation of the macrophages.[23] Our results suggest that elevated production of IFN-γ together with the low production of IL-4 in lesions of ATL indicate an induction of protective immunity and therefore of the injuries, since Th1 response is capable of activating macrophages via IFN-γ, which allows the production of reactive oxygen and nitrogen, primarily nitric oxide (NO), which facilitates the elimination of macrophages infected with amastigotes of the parasite.[21]

This work also analyzed the involvement of effector cells and regulatory cytokines. By comparing the expression of the OX40 protein with cytokines the IFN-γ and IL-4, we observed a statistically significant difference between this protein and IL-4. It is believed that this pattern of immune response observed in the lesions of ATL patients progressed to a Th1 response, which is characterized by the synthesis of IFN-γ. It appears that a predominant Th1 response is necessary for parasite control and wound healing, whereas the presence of IL-4, and probably other regulatory cytokine such as IL-10, may contribute to the escape of the parasite, and hence the persistence of an inflammatory lesion.[23,24]

CONCLUSION

From this study we noticed that high OX40 immunoreactivity may be associated with a proliferation of T lymphocytes, whereas OX40 is a protein preferentially expressed on CD4+ T cells. The present study indicates that the immune response in lesions of ATL is associated with a healing process, which can be explained by the high expression of the IFN-γ cytokine which works in infection control and disease cure.[25] In addition, we observed a low expression of IL-4, which is a cytokine that contributes to the prevention of scarring in skin diseases.[26] ❑

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