# Analysis of chemokine receptors on the surface of circulating leukocytes of individuals infected with *Mycobacterium leprae*: preliminary results

## Análise de receptores de quimiocinas na superfície de leucócitos circulantes de indivíduos infectados pelo *Mycobacterium leprae*: resultados preliminares

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**ABSTRACT**

In this study, the expression of chemokine receptors on the surface of circulating leukocytes was determined using flow cytometry. An increase in the percentage of CCR2+CD4+ lymphocytes was observed in the peripheral blood of leprosy patients. This preliminary data suggests that alterations occur in the chemokine receptor profile of these patients.

***Key-words:*** Leprosy. Chemokine receptors. Leukocytes. Flow cytometry.

**RESUMO**

Neste estudo, a expressão de receptores de quimiocinas na superfície dos leucócitos circulantes foi feita pela citometria de fluxo. Houve aumento da porcentagem de linfócitos CCR2+CD4+ no sangue periférico dos pacientes com hanseníase. Este resultado preliminar sugeriu alteração do perfil dos receptores de quimiocinas desses pacientes.

***Palavras-chaves:*** Hanseníase. Receptores de quimiocina. Leucócitos. Citometria de fluxo.

Leprosy (or Hansen's disease as it is commonly known in Brazil) is a chronic, infectious disease caused by *Mycobacterium leprae* that often leads to the development of skin lesions and nerve damage through an inflammatory immune response. Infected patients may evolve towards spontaneous cure or a wide pathological spectrum determined by elements of cellular and humoral immune response[10]. Tuberculoid patients present a vigorous immune response mediated by tissue cells and test positive for delayed type hypersensitivity (DTH) in response to *Mycobacterium leprae* antigens. On the other end of the spectrum, Virchowian patients exhibit an ineffective cellular response against *Mycobacterium leprae* associated with a high bacterial load, as observed in their bacteriological indexes (BI). Therefore, the pathogenic mechanisms of leprosy and the capacity for spontaneous cure in the face of *Mycobacterium leprae* infection are directly related to the immune response triggered in the host. The cellular response plays a protective role while the humoral response allows considerable bacterial proliferation, which is a well-established susceptibility factor[2]. In this context, the production of cytokines in active lymphocytes and monocytes, as well as the differentiated production of chemokines at the site of lesions, seem to contribute to immune response and the establishment of different clinical aspects of the disease.

Chemokines are chemotactic cytokines that recruit specific groups of leukocytes and are involved in many chronic inflammatory processes[3]. These molecules bond to cells by way of G protein-coupled receptors, classified as CXCR, CCR, CR and CX3CR. Each chemokine receptor presents a distinct, albeit overlapping, specificity for different chemokines[1]. Studies have shown that the production of TNF-$\alpha$ in monocytes and IFN-$\gamma$ in lymphocytes favor cellular interaction in affected tissue, as well as the activation and regulation of chemokines produced by epithelial cells, mastocytes, monocytes and neutrophiles, such as the CC (CCL2, CCL3 and CCL4) and CXC chemokines, as well as CXCL8, CXCL9 and CXCL10[1,3]. The presence of elevated serum levels of CXCL8[4] and CCL2[7] in Virchowian patients and the

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increased expression of chemokines CCL2 and CCL5 in the skin lesions of leprosy patients[6] suggest the participation of these factors in complex immunopathological disease mechanisms. Although the participation of chemokines and their receptors in these processes has been the focus of numerous studies, little research has been conducted regarding the profile of chemokine receptors in specific leukocytes populations and subpopulations in the peripheral blood of leprosy patients. In light of this fact, this study aimed to evaluate the expression of CXCR4, CCR2 and CCR5 receptors in populations and subpopulations of circulating leukocytes in leprosy patients compared to healthy, not-infected individuals.

In this study, six leprosy patients were evaluated clinically by the Family Health Program in Diamantina, State of Minas Gerais. The diagnosis and classification of leprosy patients was based on clinical criteria, using the standard operational classification promoted by the World Health Organization (WHO)[14]. The control group consisted of five healthy, not-infected individuals. This study was approved by the Research Ethics Committee of the Federal University of the Jequitinhonha and Mucuri Valleys and all participants signed free informed consent forms.

For analysis of the phenotypical profile of circulating leukocytes, 5ml of venal blood was collected using EDTA as an anticoagulant. After collection, part of the blood was used for a standard hemogram and the analysis of chemokine receptors in leukocyte subpopulations, followed by flow cytometry analysis. In accordance with the protocol, 0.1ml of blood was incubated with 1µl of specific monoclonal antibody for receptors of the main cellular populations of peripheral blood, such as CD3, CD4, CD8, CD19, CD16 and CD14 (R&D Systems, USA), as well as for the chemokine receptors CCR2, CCR5 and CXCR4 (R&D Systems, USA). Subsequently, the samples were submitted to red blood cell lysis with readings performed in a FACScan Flow Cytometer (Becton-Dickinson, USA). The data analysis was conducted using Cell-Quest software (Becton-Dickinson, USA). Statistical differences between the groups were evaluated by the Mann Whitney U test, established at 95% ($p < 0.05$) confidence interval.

The percentage analysis of the leukocyte populations and subpopulations in peripheral blood of leprosy patients (LE) and in not-infected individuals (NI) is represented in **Table 1**. According to the data, an increase in the percentage of NKT lymphocytes in the peripheral blood of HA patients was observed when compared to the control group NI ($p<0.05$).

**TABLE 1**

Analysis of leukocyte populations and subpopulations in the peripheral blood of leprosy patients and not-infected individuals.

| | Leprosy patients | Not-infected individuals |
|---|---|---|
| Leukocytes/mm$^3$ | 7220 ± 417 | 7311 ± 2786 |
| % monocytes | 3.0 ± 1.0 | 3.0 ± 1.0 |
| % neutrophiles | 53.0 ± 9.0 | 50.0 ±17.0 |
| % eosinophiles | 6.0 ± 4.0 | 8.0 ± 7.0 |
| % lymphocytes | 38.0 ± 5.0 | 33.0 ±11.0 |
| % B (CD3-CD19+) lymphocytes | 8.4 ± 2.3 | 10.8 ± 5.2 |
| % NK (CD3-CD16+) lymphocytes | 12.5 ±10.5 | 10.6 ± 6.3 |
| % NKT (CD3+CD16+) lymphocytes | 0.3 ± 0.2 | 2.3 ± 1.3* |
| % T (CD45+CD3+) lymphocytes | 74.6 ± 9.3 | 63.4 ± 10.2 |
| % CD4 (CD3+CD4+) T lymphocytes | 20.4 ± 9.6 | 10.6 ± 9.8 |
| % CD8 (CD3+CD8+) T lymphocytes | 44.3 ± 15.0 | 50.4 ± 25.0 |

* $p< 0.05$

**Figure 1** shows the analysis of CXCR4, CCR2 and CCR5 chemokine receptors on the surface of CD4 and CD8 T lymphocytes and the results are expressed as the median of the percentage of chemokine receptor-positive cells examined. According to the results, a statistically significant increase occurred in the percentage of CD4+CCR2+ T lymphocytes in the HA group in comparison with the NI group (NI=3.5 ± 2.2; LE=10.2 ± 3.6). Although a reduction in the percentage of

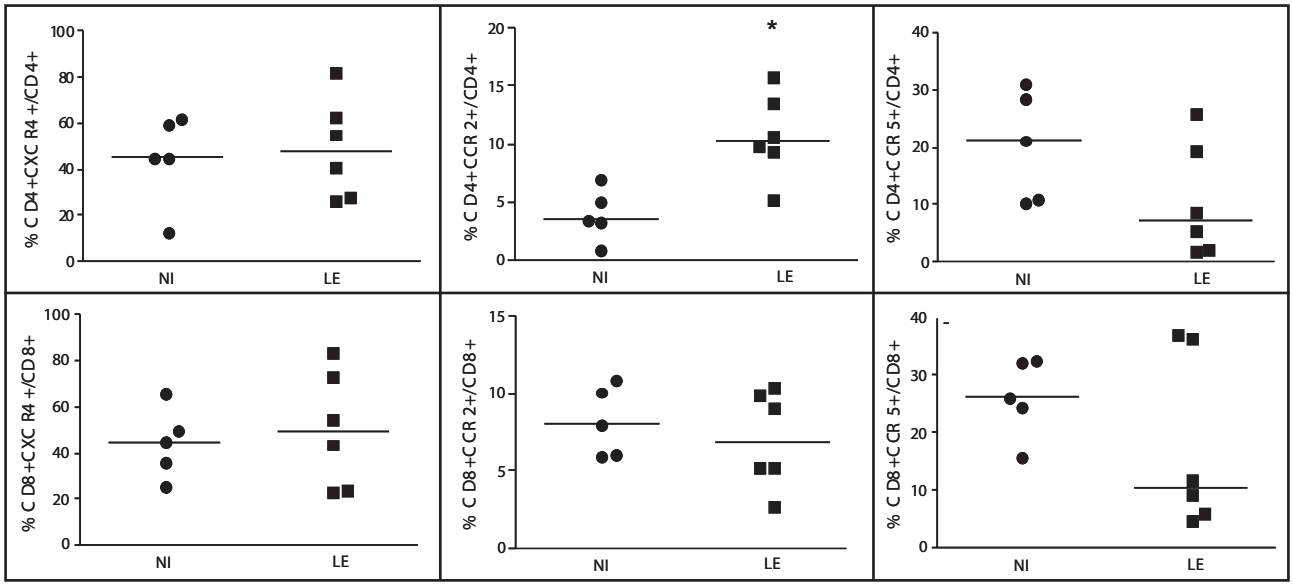


**FIGURE 1**

Percentage analysis of CD4+ and CD8+ T lymphocyte for CXCR4, CCR2 and CCR5 chemokine receptors in leprosy patients (LE=6) and in not-infected individuals (NI=5). * $p< 0.05$

CCR5+ cells was observed in the CD4 and CD8 T lymphocyte subpopulations in the LE group relative to the NI group, this difference was not statistically significant. There was no statistical difference in the analysis of CXCR4 receptors in both T lymphocyte subpopulations.

Considering the different clinical forms of leprosy promoted by the pattern of host immune response against *Mycobacterium leprae* antigens and the constant recruitment of cells to the lesions, the overriding goal this study was to analyze the phenotypical profile of leukocytes in blood compartments of leprosy patients. The majority of studies are conducted using skin or nerve tissues and use immunohistochemical analysis to determine cellular infiltration and the expression of molecules involved in the elimination process of the infectious agent[6 11]. However, few studies have investigated the phenotypical profile of cell populations and subpopulations or the production de chemokines and their receptors in leukocytes of peripheral blood cells. In this study, an increase in the percentage of cells with a phenotype suggestive of NKT lymphocytes (CD3+CD16+) was observed in peripheral blood of leprosy patients. According to data in the literature, the cellular infiltration present in skin lesions and nerves is predominantly mononuclear, which, depending on the type of cellular and humoral immune response, may lead to the formation of granulomas. The production of TNF-$\alpha$ by monocytes and IFN-$\gamma$ by CD4 T lymphocytes of the infiltrated cell interferes with intercellular processes and the production/regulation of chemokines. Some studies report that NKT lymphocytes are recruited in the face of production of chemotactic factors by epithelial cells and activated by lipoprotein antigens and glycolipids of *Mycobacterium leprae*[9 12 13]. These results corroborate the increase of circulating NKT cells observed in this study.

Hasan *et al* suggest that the differential activation of chemokines in leprosy patients appears to be critical in the dissemination and progression of the disease[5]. The analysis of differentiated production of serum chemokines has grown in recent years[4 5 7]. Mendonça *et al* demonstrated the potential of the chemokine eotaxin as a biological marker in leprosy. According to their work, leprosy patients that showed an increase in plasmatic levels of eotaxin/CCL11, where levels reached 275pg/ml, presented a sensitivity of 90% and a specificity of 95% in the detection of leprosy patients. The authors suggest that the future dosage of the marker should be used as an additional resource for disease diagnosis[8]. Research has shown that where an increase in CCL2 (MCP-1) serum levels occurs in Virchowian patients[5 7], with a corresponding rise in bacterial load, the secreted antigens may stimulate diverse cell populations in different organs and tissues. This activation appears to result in an increase in the intensity of the innate immune response, as witnessed by the elevated levels of CCL2 in Virchowian leprosy patients[5]. CCL2 promotes chemotaxis in monocytes, immature dendritic cells and in memory T lymphocytes by means of its interaction with the CCR2 receptor on the surface of these cells. In addition, the participation of this chemokine has been shown in the establishment of mononuclear infiltration in chronic inflammatory processes[3]. The present results show that an increase in the percentage of CD4+CCR2+ T lymphocytes suggests that, while this pathogen is an intracellular mycobacterium, the recruitment of helper T lymphocytes appears to occur preferentially relative to cytotoxic T lymphocytes. The use of flow cytometry was chosen in this study because, in contrast with the immunohistochemical method, this experimental strategy permits specific, sensitive and simultaneous evaluation of the expression of diverse membrane receptors, linked to the complex mechanisms of cellular recruitment, which allows for inference concerning the migratory potential in specific cell populations and subpopulations. The data presented in this article reflect a preliminary analysis, where the number of patients studied was a limiting factor. Additional studies are being conducted by our research group with the objective of increasing the body of knowledge regarding the patterns of chemokine receptor expression in the populations and subpopulations of leukocytes in the peripheral blood of leprosy patients.

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