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Original article

Characterization of inflammatory markers associated with systemic lupus erythematosus patients undergoing treatment



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ABSTRACT

Objective: To characterize the inflammatory profiles of patients with systemic lupus erythematosus receiving standard treatment compared to healthy controls.

Patients and methods: Peripheral venous blood was collected from systemic lupus erythematosus patients (n=14) and controls (n=18) at enrollment. Blood samples were used for quantification, by flow cytometry, of CD11b (integrin) and Chemokine receptor CXCR2 expression surface antigen in neutrophils and lymphocytes, while cytokines were assayed in serum samples. Purified neutrophils were assayed by their ability to phagocytize human plasma-opsonized zymosan.

Results: Patients had a median (interquartile range) disease activity index of 1.0 (0–2.0) characteristic of patients in remission. Interleukin-6 and interleukin-10 serum concentrations were significantly higher in the patient group compared to controls and the phagocytic index of circulating neutrophils was significantly reduced in patients compared to controls. The levels of interleukin-2, interleukin-5, interleukin-8 and tumor necrosis factor alpha did not significantly differ between patients and controls. Flow cytometric analysis revealed that the integrin expression levels were reduced in lymphocytes (but not in neutrophils) obtained from systemic lupus erythematosus patients, while surface expression of the chemokine receptor 2 was similar in both neutrophils and lymphocytes.

Conclusion: Systemic lupus erythematosus patients receiving standard treatment presented with elevated systemic levels of interleukin-6 and interleukin-10, reduced neutrophil phagocytic capacity, and reduced lymphocyte expression of integrin even when symptoms were in remission. These alterations to innate immune components may put these individuals at a greater risk for acquiring infections.

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Caracterização de marcadores inflamatórios associados a pacientes com lúpus eritematoso sistêmico em tratamento

R E S U M O

Palavras-chave:

Lúpus eritematoso sistêmico
Citocinas
Fagocitose
Integrina
CXCR2

Objetivo: Caracterizar os perfis inflamatórios de pacientes com lúpus eritematoso sistêmico que estavam recebendo tratamento padrão em comparação com controles saudáveis.

Pacientes e métodos: Coletou-se o sangue venoso periférico de pacientes com LES ($n = 14$) e controles ($n = 18$) no momento da entrada no estudo. As amostras de sangue foram usadas para quantificação, por citometria de fluxo, da expressão dos antígenos de superfície CD11b (integrina) e do receptor de quimiocina CXCR2 em neutrófilos e linfócitos, enquanto as citocinas foram avaliadas em amostras de soro. Avaliou-se a capacidade dos neutrófilos purificados de fagocitar zimosan opsonizado com plasma humano.

Resultados: Os pacientes apresentavam uma pontuação mediana (intervalo interquartil) no índice de atividade da doença de 1,0 (0-2,0), característica de pacientes em remissão. As concentrações séricas de interleucina 6 e interleucina 10 foram significativamente maiores no grupo de pacientes em comparação com os controles; o índice de fagocitose de neutrófilos circulantes estava significativamente reduzido nos pacientes em comparação com os controles. Os níveis de interleucina 2, interleucina 5, interleucina 8 e fator de necrose tumoral alfa não diferiram significativamente entre pacientes e controles. A análise da citometria de fluxo revelou que os níveis de expressão da integrina estavam reduzidos nos linfócitos (mas não nos neutrófilos) obtidos de pacientes com lúpus eritematoso sistêmico, enquanto a expressão do receptor de superfície de quimiocina era semelhante em neutrófilos e linfócitos.

Conclusão: Os pacientes com lúpus eritematoso sistêmico recebendo tratamento padrão apresentaram níveis sistêmicos elevados de interleucina 6 e interleucina 10, redução na capacidade fagocítica dos neutrófilos e expressão reduzida da integrina em linfócitos, mesmo quando os sintomas estavam em remissão. Essas alterações nos componentes da imunidade inata podem colocar esses indivíduos em maior risco de adquirir infecções.

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease involving both T-cell and B-cell abnormalities characterized by the loss of tolerance to nuclear self antigens and the production of autoantibodies that cause inflammation and damage to multiple organ systems.¹ An increasing array of cytokine abnormalities has been implicated in either the pathogenesis of SLE or as secondary markers that reflect immune dysregulation.^{2,3}

One of the characteristics of chronic inflammation is dysregulation of the chemokine network, including changes to the expression profiles of CXCR2 and CXCR3. CXCR2 is a G-protein coupled receptor belonging to the CXC chemokine family that serves as a receptor for IL-8 (CXCL8) and CXCL1 that mediates neutrophil recruitment, cell proliferation, and angiogenesis.^{4,5}

The CD11b/CD18 integrin (Mac-1, complement receptor 3) is a surface receptor expressed by monocytes, macrophages, neutrophils, dendritic cells, and B cell subsets that binds several ligands including members of the ICAM family and the complement factor iC3b. It is involved in essential immunological processes including leukocyte extravasation and phagocytosis. A single nucleotide polymorphism in the integrin extracellular domain resulting in an amino acid change represents one of the strongest genetic risk factors associated with human SLE.^{6,7}

The variant form is associated with reduced cell adhesion to ICAM-1, ICAM-2, and iC3b and with impaired phagocytosis, suggesting that this polymorphism (that affects the efficient removal of apoptotic cells) is associated with disease pathology. Changes to phagocytosis efficacy are not restricted to CD11b⁺/CD18⁺ macrophages but also to other phagocytic cells such as monocytes and neutrophils.⁷ Furthermore, this polymorphism is associated with impaired regulation of inflammatory cytokine networks possibly associated with SLE disease progression.^{6,8}

The impaired leukocyte function (i.e., monocytes and neutrophils) observed in SLE patients places these individuals at increased risk for acquiring bacterial infections associated with increased rates of morbidity and mortality.⁹ In addition to decreased phagocytosis of microbial pathogens, impaired clearance of apoptotic cells negatively impacts tissue homeostasis by exposing the immune system to intracellular components that are pro-inflammatory and immunogenic, thereby contributing to the development of chronic inflammation and autoimmune disorders.¹⁰

Treatment with prednisolone failed to modify the CD11b expression on peripheral blood neutrophils in patients with ulcerative colitis compared with controls.¹¹ However dexamethasone inhibited membrane, but not intracellular, expression of CD11b on murine eosinophils, associated to reduced cell chemotaxis.¹² Considering the CD11b polymorphism in LES patients and possible impairment of corticoids

on functional leukocyte capacity, patients with LES under treatment may be susceptible to functional deficit in circulating immune cells.

The present study was designed to further characterize the inflammatory response presenting in SLE patients undergoing treatment by comparing the systemic cytokine response, neutrophil phagocytic capacity, and the CD11b and CXCR2 neutrophil and lymphocyte expression profiles in both patients and controls.

Materials and methods

Patients

Women referred by the public health system to the Outpatient Rheumatology Service of the Federal University of Triângulo Mineiro (UFTM) were prospectively diagnosed with SLE according to guidelines established by the American College of Rheumatology and enrolled in the study. Patients had different times of SLE onset and were being treated with corticoids and/or hydroxychloroquine and/or azathioprine. Disease severity was established using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score system. Healthy female volunteers served as controls. The study protocol was approved by the UFTM Committee on the Use of Human Subjects, and written informed consent was obtained from all patients and controls.

Blood collection

Peripheral venous blood samples were collected from patients and controls at enrollment. At each collection time two samples (5 mL each) were collected (with or without 100 IU/mL heparin). Sera were obtained by centrifuging ($180 \times g$) the blood for 15 min. Respective serum samples were then collected and stored at -70°C until use. The assays were performed at the Laboratory of the Discipline of Pharmacology and at the Oncological Research Institute (IPON)/Discipline of Gynecology and Obstetrics.

Quantification of serum cytokine levels

The serum levels of TNF- α , IL-2, IL-5, IL-6, IL-8, and IL-10 were determined by ELISA. Briefly, flat-bottomed 96-well microtiter plates were coated with antibodies (1–3 $\mu\text{g}/\text{mL}$ in 50 $\mu\text{L}/\text{well}$) specific for each cytokine in binding buffer and incubated overnight (4°C). Plates were then washed with phosphate buffered saline-Tween-20 (PBST) and nonspecific binding blocked by incubating wells with 100 $\mu\text{L}/\text{well}$ PBS/1% bovine serum albumin (BSA) for 120 min at 37°C . Samples and standards were added to respective wells (50 $\mu\text{L}/\text{well}$) and incubated overnight (4°C). Plates were washed with PBST followed by the addition of the appropriate biotinylated monoclonal anti-cytokine antibody. After 1 h, plates were washed and avidin peroxidase (diluted 1:5000) added to each well. After 30 min the plates were washed and substrate (100 μL o-phenylenediamine dihydrochloride [OPD, Sigma, St. Louis, MO]) was added. The plates were subsequently incubated at room temperature for 15 min before H_2SO_4 (50 μL , 1M) was

added to each well to stop the reaction and the optical density measured at 490 nm using a multiwell plate reader (Multi-skan MCC340 MKII, Flow Laboratories). Data are expressed in picograms of cytokine/mL serum. Cytokine concentrations in serum samples were determined from the standard curves generated.

CD11b and CXCR2 expression by neutrophils and lymphocytes

CD11b and CXCR2 expression levels were quantified using a FACSCalibur flow cytometer and CellQuestTM software (Becton Dickinson, San Jose, CA), with acquisition of 10,000 cells in each experiment. Phycoerythrin (PE)-conjugated anti-CD11b antibody, PE/Cy5-conjugated anti-CXCR2 antibody (BD Pharmingen, San Diego, CA), and control antibodies (PE- or PECy-5-conjugated IgG_{2b}; BD Pharmingen, San Diego, CA) were used. Data are expressed as the number of absolute or the percentage of neutrophils and lymphocytes positive for CD11b and/or CXCR2 expression.

Phagocytosis assay

The phagocytosis index of neutrophils ($2 \times 10^6/\text{mL}$) harvested from either SLE patients or healthy controls was assessed by their ability to phagocytize human plasma-opsonized zymosan (10 particles/cell) over a 1 h period at 37°C (5% CO_2). The phagocytic index is expressed as follows: % of phagocytic cells \times number of interiorized particles.

Statistical analysis

Statistical analyses were performed using SigmaStat 2.03 software. Distribution was evaluated using the Kolmogorov-Smirnov test and differences between two unpaired groups (SLE patients vs. controls) were determined using the Student's *t* test or the Mann-Whitney test according to normal or not normal distribution of the data, respectively. Statistical significance was set at $p \leq 0.05$.

Results

Study population

Fourteen women diagnosed with SLE (mean time with disease \pm SD of 7.1 ± 4.3 y), mean age (\pm SD) of 40.6 ± 9.6 y (range, 24–55) and 18 healthy women, mean age of 35.3 ± 9.8 y (range, 26–55) were enrolled into the study (Table 1). Age did not differ between patients and controls ($p = 0.132$, Student's *t* test). The median (and interquartile) SLEDAI score was 1.0 (0–2.0), indicating that SLE was in clinical remission. In eight patients (57.2%) the SLEDAI score was zero, three patients (21.4%) had a score of 2, two (14.3%) patients had a score of 4 (moderate disease activity), and one patient (7.1%) had score of 9 (moderate activity) (Table 1).

The treatment received by SLE patients at enrollment is described in Table 1. Three (21.4%) patients were treated with only corticoids (prednisone or prednisolone), 2/14 (14.3%) received hydroxychloroquine, 6/14 (42.9%) received both

Table 1 – Systemic lupus erythematosus (SLE) patient characteristics and disease parameters.

Number of patients	14
Average age \pm SD, years	40.6 \pm 9.6
Age range	24–55
Sex (female/male), n	14/0
Disease duration (mean \pm SD), years	7.1 \pm 4.3
Disease duration range, years	1–15
SLEDAI score, medians (25–75%)	1.0 (0–2.0)
SLEDAI score, n (%)	
Zero	8 (57.2%)
2	3 (21.4%)
4	2 (14.3%)
9	1 (7.1%)
Treatment, n (%)	
Corticoids	3 (21.4%)
Hydroxychloroquine	2 (14.3%)
Corticoids plus hydroxychloroquine	6 (42.9%)
Corticoids or hydroxychloroquine plus azathioprine	3 (21.4%)

Table 2 – SLE and control serum cytokine concentrations (pg/mL).

Cytokines (pg/mL)	Controls (n=18)	Patients (n=14)
IL-2	9.6 (5.3–19.1)	7.3 (6.5–12.8)
IL-5	11.6 (4.5–25.1)	4.4 (2.8–7.7)
IL-6	3.9 (2.6–5.2)	6.8 (5.2–8.5) ^a
IL-8	13.2 (11.5–23.6)	21.3 (17.2–31.3) ^b
IL-10	0.0 (0.0–4.5)	13.5 (0–17.2) ^c
TNF- α	0.0 (0.0–3.5)	1.3 (0.2–3.1)

Data are expressed as medians (interquartile range).

^a $p < 0.01$.

^b $p = 0.115$ compared to the control group (Mann–Whitney test).

^c $p < 0.05$.

corticoids and hydroxychloroquine, and 3/14 (21.4%) received azathioprine plus corticoid or hydroxychloroquine.

Serum cytokine concentrations

Serum cytokine levels were characterized in both patients and controls. The median serum levels of TNF- α , IL-2, and IL-5 did not differ significantly between groups (Table 2). IL-8 levels were elevated in SLE patients compared to controls but the levels were not statistically significant ($p = 0.115$). But the serum concentrations of IL-6 ($p < 0.01$) and IL-10 ($p < 0.05$) were significantly higher in SLE patients at enrollment compared to controls (Table 2).

CD11b and CXCR2 expression by neutrophils and lymphocytes

The surface expression of CD11b and CXCR2 by neutrophils and lymphocytes harvested from patients ($n = 14$) and a subset of randomly selected controls ($n = 16$) was assessed by flow cytometry. The absolute number of lymphocytes harvested from SLE patients expressing CD11b was lower compared to expression levels observed in controls (Fig. 1C). No differences in CD11b expression by neutrophils were detected (Fig. 1A and B). The number of CXCR2 positive cells did not differ in

either neutrophils (Fig. 2A and B) or lymphocytes (Fig. 2C and D) harvested from patients and controls.

Phagocytosis assay

The ability of neutrophils harvested from patients ($n = 14$) and controls ($n = 18$) to phagocytize opsonized zymosan was evaluated. Neutrophils from SLE patients presented with a significantly lower median (interquartile range) phagocytic activity index, 178.0 (49.0–304.0) compared to control neutrophils, 577.0 (209.0–1131.0) ($p = 0.017$, Mann–Whitney).

Discussion

The present study defined the inflammatory cytokine profile of SLE patients undergoing therapy. As expected, all patients enrolled were women since SLE is nine times more common in women than in men.¹ Increased serum levels of IL-6 and IL-10 were detected in SLE patients compared to controls although the levels of TNF- α , IL-2, IL-5 and IL-8 did not differ between groups.

The nucleosome is a major autoantigen that can be detected as a complex in the serum of SLE patients with the potential of directly activating neutrophils.¹³ This was confirmed by incubating neutrophils from healthy individuals with plasma obtained from SLE patients that responded by producing increased levels of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-8.¹⁴

IL-6 is likely to be involved in autoantibody production in SLE patients.³ Data presented in this report demonstrated significantly elevated IL-6 serum levels in SLE patients compared to controls, according to other studies.^{15,16} It was reported that the levels of IL-6 correlated with the IL-12 and IL-23 levels observed at the time of SLE diagnosis and after prednisone treatment was initiated.¹⁵

Serum IL-8 levels were elevated in lupus patients but not significantly. SLE patients with active or inactive disease presented with elevated levels of chemoattractant proteins, including IL-8, compared to controls, regardless of the drug treatment regimen used and the degree of tissue damage.¹⁷ The authors suggest that long-term treatment of SLE with standard immunomodulatory drug regimens fails to normalize the levels of key chemoattractant proteins linked to innate immunity and that a baseline pro-inflammatory state persists in SLE patients.¹⁷

High systemic concentrations of chemokines such as IL-8 have the potential of inducing receptor desensitization and rendering leukocytes unresponsive to subsequent exposure.¹⁸ Our present data did not identify differences in the CXCR2 expression levels on circulating neutrophils or lymphocytes, maybe, in part, because serum IL-8 levels did not reach higher significant levels in patients compared to controls. But other studies have demonstrated that neutrophils from SLE patients had decreased CXCR2 gene expression levels, compared to controls, leading to IL-8 hypo-responsiveness *in vitro*.⁵ The CXCR2 expression levels in T cells were increased in patients with active SLE compared to patients with inactive disease and to healthy controls.⁴

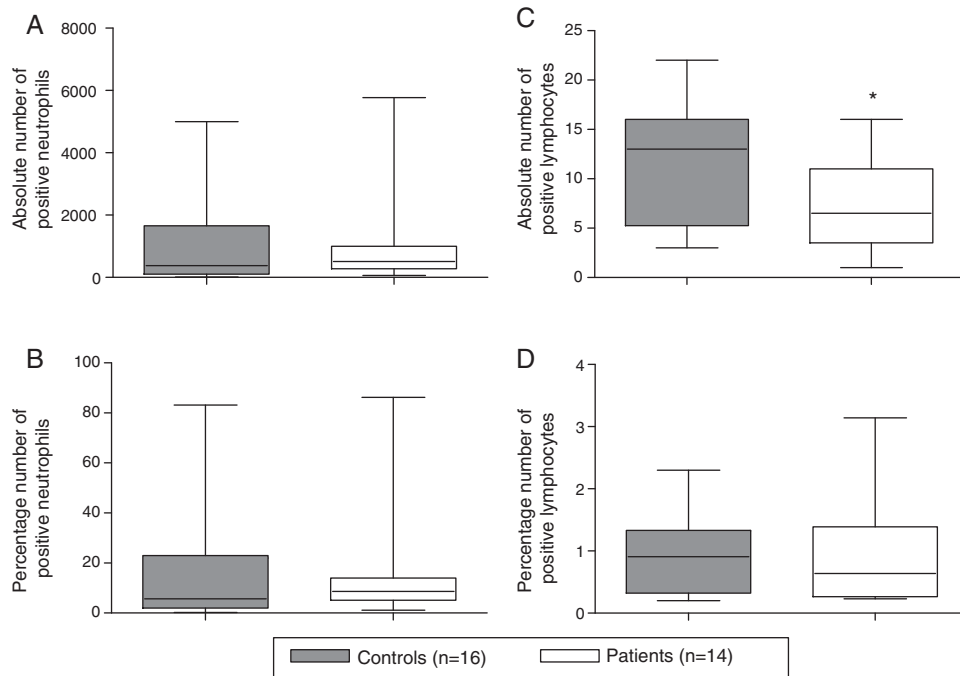


Fig. 1 – Analysis of CD11b expression. Neutrophils obtained from either controls ($n = 16$) or Systemic lupus erythematosus (SLE) patients ($n = 14$) under treatment were analyzed by flow cytometry. The absolute numbers (panels A and C) and percentages (panels B and D) of neutrophils (panels A and B) and lymphocytes (panels C and D) positive for CD11b are shown. The 25th and 75th percentiles are represented by a bar centered around the median. The minimum and maximum values are represented by error bars. * $p < 0.05$ compared to the respective control (Student's t test).

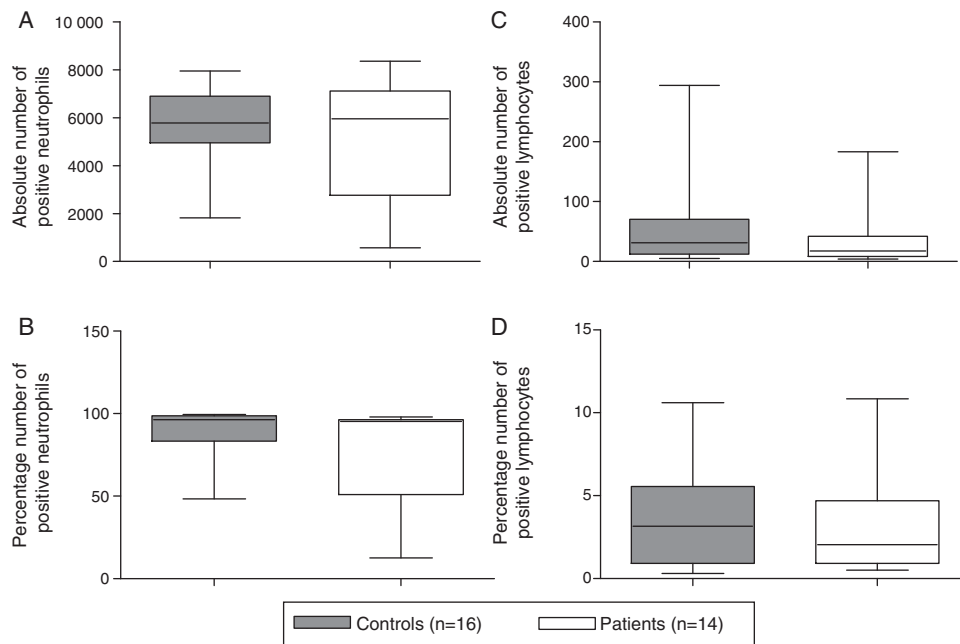


Fig. 2 – Analysis of CXCR2 expression. Neutrophils obtained from either controls ($n = 16$) or SLE patients ($n = 14$) under treatment were analyzed by flow cytometry. The absolute numbers (panels A and C) and percentages (panels B and D) of neutrophils (panels A and B) and lymphocytes (panels C and D) positive for CXCR2 are shown. The 25th and 75th percentiles are represented by a bar centered around the median and the minimum and maximum values are represented by error bars.

The role of IL-10 in SLE is somewhat controversial. IL-10 is generally considered an anti-inflammatory and an immunosuppressive cytokine. However, it is overexpressed in lupus patients and in some cases has been reported to act as a lupus-promoting molecule.² Several studies have demonstrated that the serum/plasma IL-10 levels were markedly increased in SLE patients compared to controls,¹⁹ as shown in the present study.

Increased IL-10 levels have been correlated with disease activity or with the production of autoantibodies that can contribute to SLE pathogenesis¹⁹ and/or modulate the differentiation and function of dendritic cells. Monocyte-derived dendritic cells activated by either SLE serum or following exposure to exogenous IL-10 had reduced expression levels of human leukocyte antigen (HLA)-DR and exhibited impaired capacity to stimulate allogenic T-cell proliferation.²⁰

Cells expressing a CD11b polymorphism displayed increased IL-6 production compared to wild type-CD11b-expressing cells and this genetic polymorphism resulted in an integrin isoform incapable of mediating cell adhesion via interactions with either ICAMs or iC3b.⁶ Data presented in this report showed that the CD11b expression profile on neutrophils was similar between SLE patients and controls; however, a reduced absolute number of lymphocytes from SLE patients expressed CD11b. This deficiency may ultimately lead to immune system malfunction and contribute to the development of SLE.⁶

The present study also detected that the neutrophil phagocytic index was reduced in SLE patients compared with controls, a finding that, associated to reduced expression of CD11b, may put these individuals at a greater risk for developing infections. Continuous exposure to nucleosomes may partly explain the observed reduction in activity associated with polymorphonuclear neutrophils (PMN) isolated from SLE patients, as well as the reduced PMN counts and the general state of inflammation observed in these individuals.¹⁴ It has been reported that lupus patients presented with low-density circulating neutrophils have an altered phagocytic potential despite their proinflammatory phenotype (defined by their cytokine profile).²¹

A study comparing the function of neutrophils among pediatric-onset SLE patients and healthy subjects demonstrated that regardless of infection status, medication, and disease severity, SLE patients had an impaired ability to phagocytize *Salmonella*-specific lipopolysaccharides compared to controls.⁹ In addition, macrophages isolated from patients with untreated SLE phagocytized apoptotic neutrophils less effectively than neutrophils harvested from healthy donors,¹⁰ thereby contributing to the development of chronic inflammation and autoimmunity.

Of note, our results demonstrate, at a same time point, associated alterations in parameters of the innate immune response, even when SLE patients were mainly with inactive disease, pointing to a potential mechanism for susceptibility to infections. But a limitation of the present study is the small number of patients. Also, although most patients had disease in remission, the study design did not evaluate the individual role of disease activity in the inflammatory profile of patients. Additional research with a larger patient population and with patient groups at the same grade of

disease activity would favor a more accurate comparison of results.

In conclusion, SLE patients receiving immunosuppressive treatment presented with elevated systemic levels of IL-6 and IL-10, reduced neutrophil phagocytic capacity, and reduced lymphocyte expression of CD11b. These associated findings were present even when symptoms were mainly in remission, suggesting that these alterations to innate immune components may put these individuals at a greater risk for developing infections.

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Conflicts of interest

The authors declare no conflicts of interest.

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