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Article

Cell phenotypes as activity biomarkers in patients with Systemic Lupus Erythematosus

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The pathogenesis of systemic lupus erythematosus (SLE) is complex. Few studies in Brazilian population have addressed cell phenotypes associated with immunological responses and their associations with SLE activity. The aim of this study is to investigate cell phenotypes associated to SLE diagnosis, treatment and activity. Twenty-eight SLE female patients (17 inactive, 11 active) and 10 healthy women were included in this study. Markers of natural killer (Nk), T and B cells in peripheral blood were evaluated by flow cytometry. Nkt cells were decreased only in SLE active patients. Activated CD4+, regulatory T FoxP3+ and B cells were decreased in both active and inactive SLE patients, compared to control group. The data corroborate the disruption of immune regulatory response in SLE patients and suggest phenotipic changes as possible biomarkers of SLE activity.

Keywords: Systemic Lupus Erythematosus. Lupus nephritis. T cells. B cells. NK cells.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is an autoimmune and multisystemic disorder resulting from loss of immunological tolerance to autoantigens and may cause damage to multiple organs (Cui, Sheng, Zhang, 2013). It is a disease with an incidence that varies from 4.8 to 8.7 per 100,000 inhabitants within the Brazilian population and affects young women during their childbearing years (Borchers *et al.*, 2010; Nakashima *et al.*, 2011).

Although the causes of SLE are not fully elucidated, abnormal functioning of the immune system has been demonstrated. There is an imbalance in immune proinflammatory and regulatory pathways. B-cells are persistently activated, producing autoantibodies, which form immunocomplexes that can deposit in organs and tissues leading to inflammation and damage (Dolff *et al.*, 2007; Mathian *et al.*, 2015).

Renal involvement is frequent and is one of the most severe manifestations of SLE increasing morbidity and mortality. Clinically evident lupus nephritis (LN) occurs in up to 50% of the patients, but biopsy studies have demonstrated some degree of renal involvement in almost all patients (Ichinose *et al.,* 2019; Iwata *et al.*, 2011).

SLE shows periods of activity and remission. SLE activity is assessed through the use of indexes that record clinical and laboratory manifestations of this disease.

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Among validated indexes for clinical practice is the Erythematosus Systemic Lupus Disease Activity Index 2000 (SLEDAI-2 K) (Gladman, Ibanez, Urowitz, 2002). The disease reflects an imbalance of the adaptive immune system involving T and B lymphocytes abnormalities, although there is evidence of the importance of innate immunity in the pathogenesis of SLE with involvement of dendritic cells, neutrophils and macrophages (Schleinitz *et al*., 2010).

Several direct and indirect evidences suggest that T cells play a crucial role in the pathogenesis of SLE (Dolff *et al*., 2007). There is increasing evidence that T-cell regulatory function is altered and contributes to the amplification and perpetuation of the immune response. Regulatory T cells (Treg), phenotypically characterized as CD4+CD25+ Foxp3+, have an important role in peripheral tolerance, suggesting a participation in the control of autoimmunity (Takeuchi *et al*., 2006). Imbalance in the adaptive response observed in the levels of helper T CD4 cells (Th) (CD3+CD4+) and regulatory CD4+ T cell deficiency (Treg) is involved in the pathogenesis of SLE. This imbalance is responsible for changes in the levels of proinflammatory cytokines observed in SLE (Dolff *et al*, 2011). The main function of CD4+ T cells is to modulate adaptive immunity through the secretion of cytokines, regulate CD8+ T (CD3+CD8+) cell response and participate in the differentiation of B cells and antibody-secreting cells (Daca *et al*., 2011).

NK cells (CD3-CD56+) are a heterogeneous group of large granular lymphocytes that do not express CD3. These may be primarily classified into two subpopulations depending on the CD56 expression density. About 90% of NK cells show low expression of CD56 (CD56dim) and are responsible for cytotoxic activity, while the remaining 10% show significant expression of CD56 (CD56bright) having the function of secreting cytokines (Crispin *et al.,* 2003). In NK cells, NKG2D is an activation receptor that recognizes several stress-induced ligands expressed by cancer and virus-infected cells, being essential for enhancing NK cytotoxicity (Boerman *et al*., 2015). Thus, we thought that it could be also involved in activation pathway in immune-mediated diseases (Rahman, Isenberg, 2008). Among the CD3+ T cells is a small NK cell fraction expressing CD56 which constitute the CD3+CD56+ population, known as NKt. Cheen *et al.,* 2015 and Green *et al*., 2007 observed reduced population of NKt in patients with SLE.

Elucidation of mechanisms that modulate the development of adaptive immunity in SLE patients is still a major challenge, which may allow a better understanding of the immune system in SLE in its different manifestations contributing to the development of new therapeutic strategies for SLE.

In this context, this study aimed to investigate phenotypes expression of T, B and NK cells differences among SLE patients with active or inactive disease, with or without LN, and healthy controls.

MATERIAL AND METHODS

Patients

Twenty-eight SLE female patients according to the revised American College of Rheumatology classification criteria (Hochberg, 1997) were included. Control group consisted of 10 healthy female volunteers, without familiar history of rheumatic diseases, matched for patients's age. Patients and controls were enrolled after signing a free and informed consent term. This study was approved by the Research Ethics Committee of Federal University of Minas Gerais (ETIC: 01928412.8.0000.5149), Brazil. Demographic (age, gender, socioecomic status) characteristics were collected using a standardized questionnaire. SLE activity was evaluated with Systemic Lupus Erythematosus (SLEDAI)-2K score. Patients with SLEDAI-2K≤ 4 were categorized as inactive (SLE-I) (n = 17), while SLEDAI-2K > 4 were classified as active (SLE-A) (n = 11). History of LN was recorded in 10 SLE patients according to previous kidney biopsies results. Exclusion criteria for both groups included diagnosis of other conditions or immunosuppressive diseases, such as HIV infection/AIDS and pregnancy.

Blood Sampling

A sample of 5 mL of peripheral blood was collected from each patient or control, with EDTA.K3 for immunophenotypic profile of T, B and NK cells.

After blood collection, samples were submitted to immunophenotyping protocol by flow cytometry.

Flow cytometric analysis of peripheral blood leukocytes

Immunophenotyping assays of the leukocytes were performed according to the protocol of the Centre for Infectious Diseases, USA. Briefly, 100 µl aliquots of peripheral total blood collected in EDTA were added to 5µl of monoclonal antibodies (mAbs) specific for human anti-CD3-APC (BD Biosciences) (T lymphocytes and NKt population), anti-CD4-FITC (Dako), anti-CD8-PE-Cy5 (BD Biosciences), (T CD4 and T CD8 lymphocytes subpopulations) anti-HLA-DR-PE (BD Biosciences) (cellular activation), anti-CD19-PE (BD Biosciences) (B lymphocytes), anti-CD56-PE (BD Biosciences) (NK and NKt cells), anti-NKG2D-PerCP-Cy5.5 (BD Biosciences) (NK cells activation), anti-FoxP3-PerCP-Cy5.5 (BD Biosciences) and CD25-PE (BD Biosciences) (T regulatory cells markers). Cell preparations containing the antibodies were incubated for 30 minutes at room temperature and protected from light. Then, sample hemolysis was performed by adding to each tube 2 ml of lysing solution (FACS Lysing Solution, Becton Dickinson). Preparations were incubated for 10 minutes at room temperature, protected from light and then subjected to centrifugation at 400g for 5 minutes. Then, leukocytes pellet was washed three times in 2 mL of Phosphate-buffered saline (PBS), pH 7.4, containing 0.5% of bovine serum albumin (BSA) and 0.1% sodium azide. Cells were then fixed using 200 mL of fixing solution (10g/L paraformaldehyde, 1% sodium cacodylate, 6.67 g/L sodium chloride, pH 7.4). After 10 minutes of fixation, tubes used for intracellular labeling were centrifuged at 400g for 5 minutes and washed again in PBS to remove the fixing solution. For detection of intranuclear marker FoxP3, 1 ml of PBS containing saponin solution (PBS, 0.5% saponin) was added to perform cell permeabilization. Subsequently, tubes were incubated for 30 minutes at room temperature, washed once in PBS containing saponin and submitted to centrifugation at 400g for 5 minutes and then supernatant discarded. A volume of 10µL of anti-FoxP3 antibody was added into each tube. After 60 minutes of incubation, cells were washed in 1 mL of PBS containing saponin and then in 1 ml of PBS only. Finally, 200µL of fixing solution were added to the tubes containing the cell suspension, and then the data were acquired on a flow cytometer.

Data acquisition was performed in an LSR Fortessa™ flow cytometer (BD, USA). At least 100,000 events were collected in each tube. For data analysis the Flow Jo™ software (TreeStar Inc.) was used, and the percentage of the cell population under study was evaluated by calculating the average value of the fluorescence for each phenotype studied.

Statistical analysis

All statistical analysis was performed by GraphPad Prism software 6™. All p values < .05 were considered statistically significant. Normal data were analyzed by T-Student test and analysis of variance (ANOVA). Non normal data were analyzed by Mann-Whitney and Kruskal-Wallis tests. To evaluate the normality of the variables the Shapiro-Wilk test was performed.

RESULTS

Patients

Twenty-eight SLE female patients with SLE, aged 18 - 69 years were recruited, whose clinical data were used to calculate the SLEDAI-2 K index which reflects the SLE activity [7]. Seventeen patients (age: 39.2; ±12.8) presented the inactive SLE form (SLEDAI-2 K ≤ 4) and eleven (age: 29.1; ±11.1) the active form (SLEDAI-2 K > 4). SLE-A group presented a disease duration of 7.0 ±7.6 years and SLE-I group of 8.1 ± 3.8 years (p=0.855; T-Student). Patients were under treatment and drugs such as azathioprine, prednisone (9,2; ±9,3mg) and hydroxychloroquine were most used by patients. Twelve patients were using azathioprine, 25 were using prednisone and 20 were using hydroxychloroquine. It was not possible to form a group of non-treated due to the limited number of patients.

Ten healthy women, with no previous or family history of SLE or autoimmunity, aged from 21 to 89

years-old (37.0, ±18.6) comprised the negative control group (NC). The age between the study groups did not show any difference when evaluated through ANOVA test (p = 0.267). Patients who developed Lupus Nephritis (LN) underwent biopsy to assess the extent and severity of renal injury. Four patients with LN were classified as having class II, four as class III and two as class IV, according to WHO/ISN/RPS criteria (Soares, Telles, Moura, 2005).

Phenotypic analysis of CD4+ and CD8+ T cells

The population of CD3+CD4+ T cells was decreased in SLE patients (SLE-I, SLE-A, with or without LN) when compared to NC group. On the other hand, CD3+CD8+ T cells expression was significantly increased in all SLE patients subgroups, and the population of activated CD8+ T cells (CD3+CD8+HLADR+) was also increased in SLE-A, with or without LN when compared to NC (Figure 1 A and B).

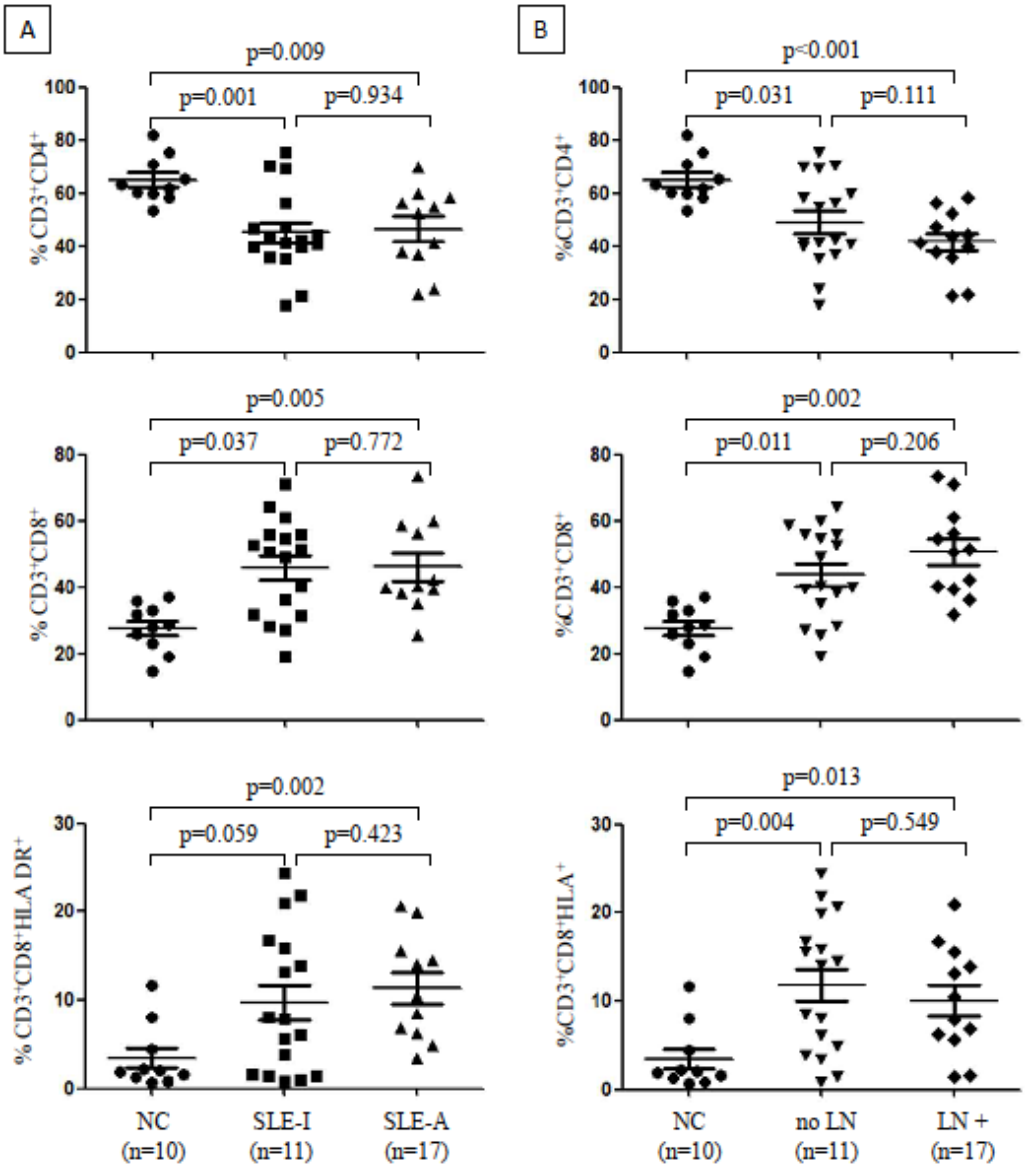

FIGURE 1 - Phenotypic expression (%) comparisons of the population of CD3+CD4+, CD3+CD8+ and CD3+CD8+HLADR+ cells among patients with inactive (SLE-I) or active (SLE-A) systemic lupus erythematosus (A), with (LN+) or without (no LN) (B) lupus nephritis and control group (NC). ANOVA, T-Student. Significance: p <0.05.

Phenotypic analysis of regulatory T cells

Analysis of regulatory T cells was performed using CD3, CD4, CD25 and FoxP3 markers. Population of CD3+CD4+CD25highFoxP3+ cells was reduced in SLE-A patients and in patients with no LN, when compared to NC (Figure 2 A and B).

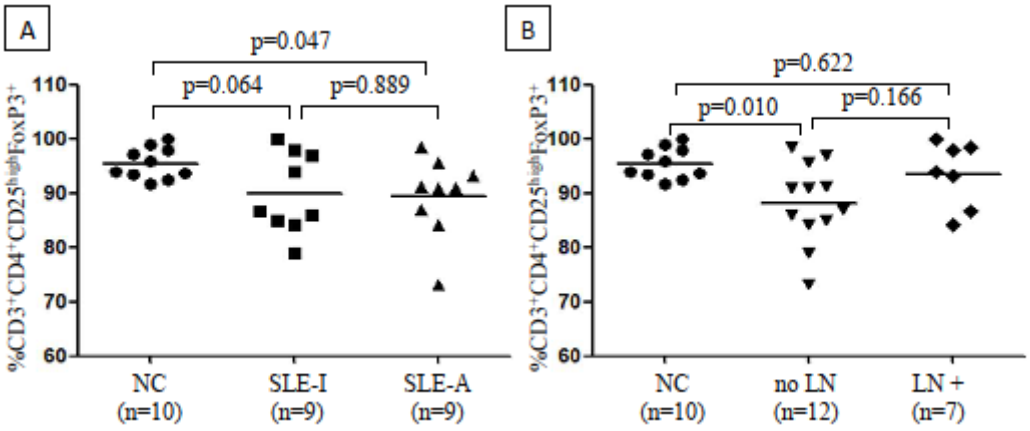

FIGURE 2 - Phenotypic expression (%) comparisons of CD3+CD4+CD25highFoxP3+ (regulatory) T cells among patients with inactive (SLE-I) or active (SLE-A) systemic lupus erythematosus (A), with (LN+) or without (no LN) lupus nephritis (B) and control group (NC). ANOVA, T-Student. Significance: $p < 0.05$.

Phenotypic analysis of B cells

Population of CD19+ B cells was significantly decreased in inactive SLE patients, when compared to NC. No difference was observed in patients presenting LN or not, suggesting that CD19+ B cells decrease in SLE is not a feature of LN.

Phenotypic analysis of NK cells

Populations of CD3-CD56+ and CD3-CD56+NKG2D+ (used as a marker of cell activation) NK cells were similar among all SLE patients groups and NC. NKt (CD3+CD56+) population, however, was decreased in patients with active SLE with LN, when compared to NC, and patients witn inactive SLE or without LN. An increased expression of NKG2D was observed after analysis of MFI of CD3+CD56-NKG2D+ in patients with LN compared to the control group (p=0.011, T-Student) and the population without LN (p=0.026, T-Student) (*data not shown*).

DISCUSSION

The complexity of the pathophysiology of SLE is consequent to changes that occur in the immune system resulting from genetic susceptibility and environmental factors. Comparison among the three groups showed that the subpopulation of NKt cells decreased only in patients with active disease. This finding is important once NKt may have a role in discriminating among those with active disease, however under treatment, which may have impact on the treatment. Populations of effector CD4+ showed decreased in patients with both inactive or active SLE compared to control group. Regulatory T cells were decreased in patients with active SLE. In contrast, the population and activation of CD8 T cells showed elevated in patients with SLE.

SLE is an autoimmune disease characterized by defects of the immune cell subsets. Despite the improved understanding of the immune response mechanisms that lead to tissue damage in SLE, the contribution of other immune cells such as T cells, NK cells, B cells and regulatory T cells, needs further studies. This study

investigated the occurrence of phenotypic alterations that could clarify and/or discriminate individuals with SLE and the status of the disease (active or inactive).

CD4 cells are thought to be the primary lymphocytes subpopulation involved in SLE autoimmune response. In the present study, a decrease in the percentage of CD3+CD4+ cells was observed in the patients group compared to the control group (Figures 1A and 1B) in accordance with previous studies (Dolff *et al.*, 2011; Le Coz *et al.*, 2013; Yu *et al.*, 2012). This abnormality could be explained by the inhibition of the maturation of CD3+CD4+ cells due to the immunosuppressive state of individuals with SLE (Li *et al.*, 2010).

Regulatory T cells (CD3+CD4+CD25+FoxP3+) are a lineage that express the transcription factor FoxP3. Studies differ according to the decrease in the number and function of these circulating cells in patients with SLE (Bijl *et al.*, 2001; Kleczynska *et al.,* 2011). Regulatory T cells have the ability to inhibit the response of autoreactive T cells and prevent exuberant immune responses directed against foreign antigens (Bijl *et al.*, 2001; Kleczynska *et al.,* 2011). Only cells with a high expression of CD25+ show potent regulatory action and are selected in flow cytometry to characterize regulatory T cells (Daca *et al.*, 2011). In the present study, we have been characterizing the population expressing the CD3+CD4+CD25highFoxP3+. The percentage of these cells was significantly lower in the peripheral blood of patients (Figure 2A). Lower cell counts were also observed in SLE patients without nephritis (Figure 2A and 2B). Decreasing in regulatory T cells promote uncontrolled progression of autoimmune diseases including SLE, since these cells have immunosuppressive properties and can modulate the development of autoimmune diseases (Tang, Bluestone, 2008), and it has been reported in several autoimmune disorders. Recently, a follow-up study by Goropevšek *et al.*, 2017, showed a more severe disease course on SLE patients who had lower-level counts of regulatory T cells**.** However, no difference was found between patients and controls for SLE patients with nephritis (Alvarado-Sánchez *et al.*, 2006; Goropevšek *et al.*, 2017). In these cases, it cannot be ruled out that inflammatory mediators produced by the damaged renal tissue itself could block the proliferation of regulatory T cells, after a certain time of disease, as demonstrated by Afzali *et al.*, 2010.

The role of cytotoxic CD8+ T cells in the defense against viral agents or organ-specific autoimmune disease is well documented (Rahman, Isenberg, 2008), but it is still unexplored in SLE. In the present study it was observed that percentage and activation of CD8+ T cells were increased in SLE patients compared to the controls. And the number of CD8+ T cells is correlated with disease activity, since it is also elevated in nephritis and may be responsible for the increased production of autoantigens (Blanco *et al.*, 2005) (Figure 1A and 1B). These findings are in agreement with other studies (Bijl *et al.*, 2001; Wouters *et al.*, 2004) and despite the important role of this cell phenotype in the defense against infectious agents, the role of CD8+ T cells in SLE is unclear, having been the object of study over the last few years.

Previous reports (Crispin *et al.*, 2010; Li *et al.*, 2010) have shown that the increase in CD8+ T cell population is due to γδ type CD8+ T cells that help in the activation of CD4+ T cells. Depletion of CD8 T cells reduces the severity of disease in experimental autoimmune glomerulonephritis (Rahman, Isenberg, 2008). The mechanism that leads to high activation of CD8+ T cells may be a consequence of the action of dendritic cells that, due to the presence of immunocomplexes, lead to a cross-presentation of these antigens causing an increase in the number of activated CD8+ T cells (Blanco *et al.*, 2005).

NK cells (CD3-CD56+) participate in innate immunity but also affect adaptive immune response. They are described as modulators of autoimmune diseases and stimulate the antibody production by B lymphocytes, but their role in the pathology of SLE is still unclear (Guo *et al.,* 2012; Schepis *et al.,* 2009). When activated, NKt cells (CD3+CD56+) represent a heterogeneous cell population, produce proinflammatory cytokines and degranulate granzymes or other cytotoxic components (Cho *et al.,* 2011). Some authors have observed a reduced population of NKt with an impaired function in patients with SLE and this reduction could be correlated with the disease activity (Cheen *et al.,* 2015; Green *et al.*, 2007). These results are in agreement with the findings of this study which has also found significantly reduced levels of NKt cells in patients with active disease. This

cellular phenotype decreasing in SLE can be attributed to defects in co-stimulatory pathways, where NKt cells activation and proliferation could be inhibited by the reduction in the expression of the co-stimulatory molecule CD26 (Green *et al.*, 2005; Green *et al.*, 2007). Therefore, additional studies could bring new insights on the various subpopulations of NKt cells and their relationship with active SLE. The data obtained in this study showed no difference in total percentage of circulating NK cells (Figure 4A and 4B), or their subpopulations, between SLE patients and the control group. This finding differs from results observed in other studies for these phenotypes which have shown decreases in patients with SLE (Ahearn *et al.,* 2012; Henriques *et al.,* 2013). According to Green *et al.,* 2005, a smaller number of circulating NK cells was observed and also with lower cytotoxic potential. Deficiency of NK cells in SLE may contribute to the changes in the immune system, but it remains unclear if these changes are related to disease activity. In the present study, we observed an increase in the expression of NKG2D on CD3+CD56- cells of patients with LN. This marker is expressed on NK cells, but can also be found in CD8+ cells and play an important role in the activation of these cells together with additional activation signals (Spada *et al.,* 2015). Greater expression of this marker in patients with lupus nephritis could indicate increased activity of these cells in these patients, and possibly contribute to renal injury. Spada *et al.*, 2015, observed an increased expression of NKG2D ligands in lupus-prone mice's glomerulus correlated to a lupus nephritic process. Moreover, no difference was observed in the expression of NKG2D markers in CD3+CD56- cells among those individuals with lupus and those without nephritis and NC.

B cells are important in the pathogenesis of SLE, since they are related to the presentation of autoantigens, production of autoantibodies and cytokines. In this context, it becomes relevant that these cells can also exert an immunosuppressive effect by secreting, for example, IL-10 capable of inhibiting differentiation of CD4+ cells. B cells are usually at low number in SLE, and we cannot rule out the existence of reactive autoantibodies against B lymphocytes that deplete them and decrease their number (Ben *et al.*, 2014; Chang *et al.*, 2008).

The expression of CD19 on peripheral blood from SLE patients was lower than in the control group and is in agreement with some reports in literature (Figure 3A e 3B) (Dolff *et al.,* 2007; Zhu *et al.,* 2018). This finding is also in accordance with lower number of T cells CD4 (Figure 1), and we must consider that these cells are responsible for B cells' activation and expansion process. Thus, this cellular phenotype, although reduced in this autoimmunity, can be a good diagnostic/prognostic marker (Zhu *et al.,* 2018).

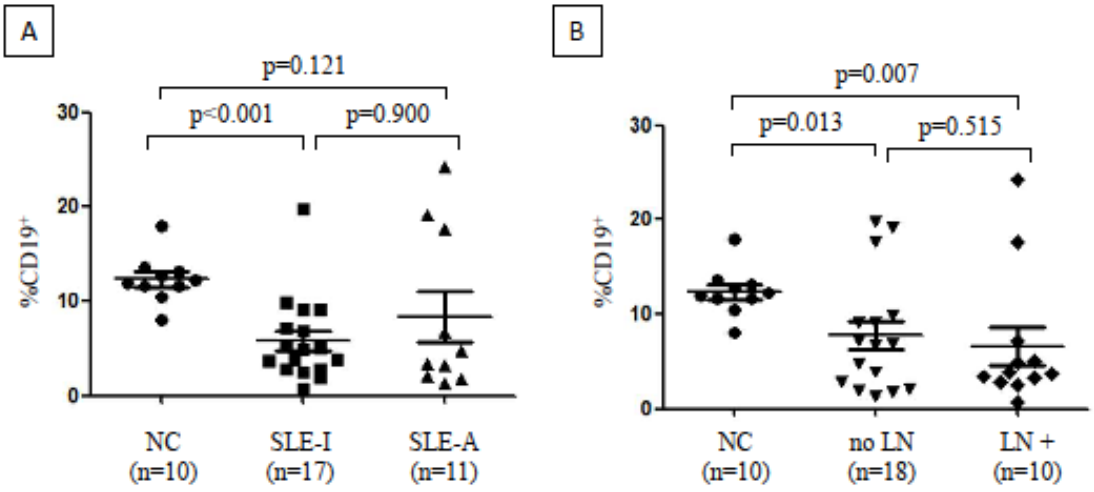

FIGURE 3 - Phenotypic expression (%) comparisons of CD19+ B cells among patients with inactive (SLE-I) or active (SLE-A) systemic lupus erythematosus (A), with (LN+) or without (no LN) lupus nephritis (B) and control group (NC). ANOVA, T-Student. Significance: $p < 0.05$.

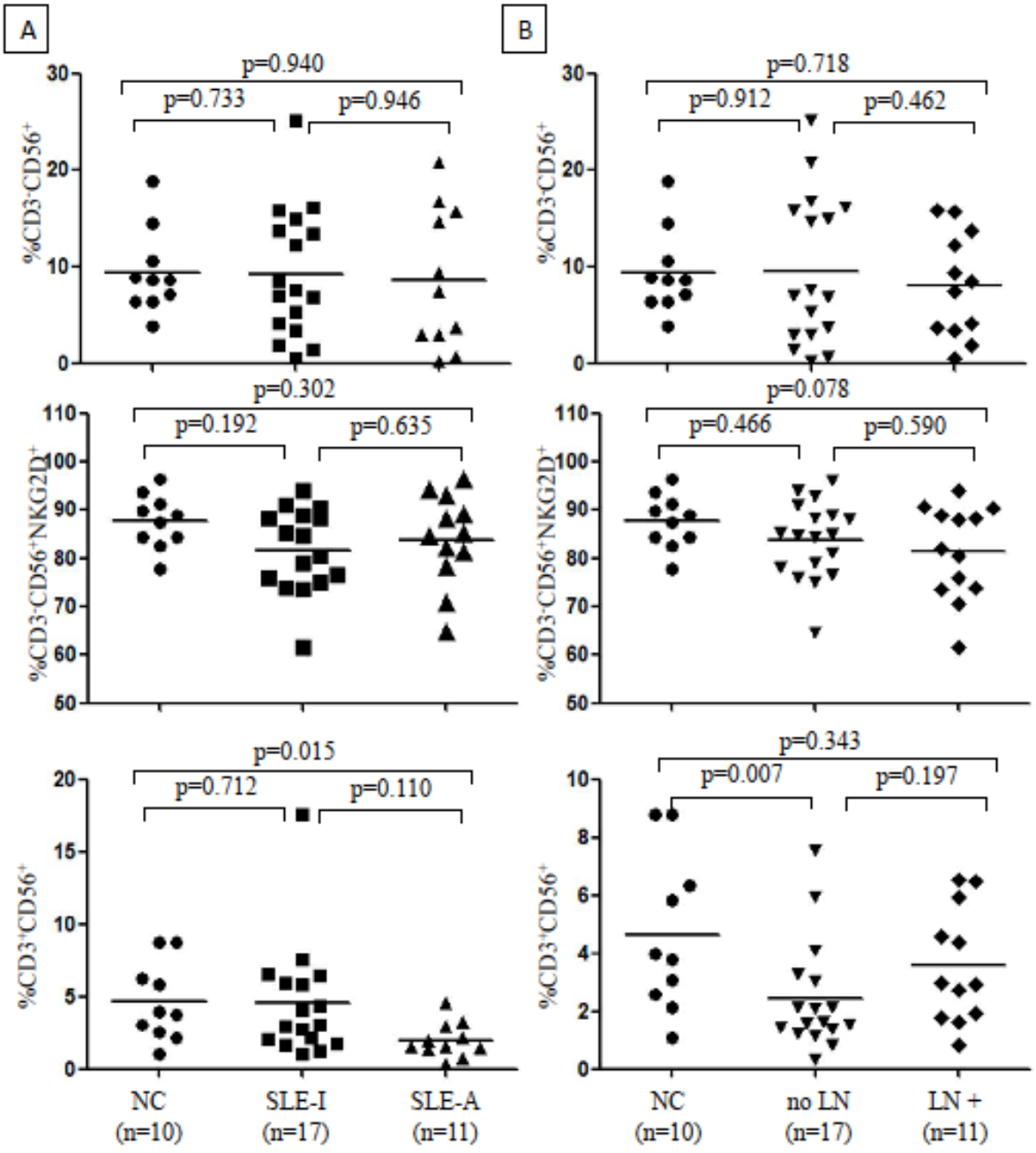

FIGURE 4 - Phenotypic expression (%) comparisons of CD3-CD56+, CD3-CD56+NKG2D+ (activated) and CD3+CD56+(NKt) NK cells among patients with inactive (SLE-I) or active (SLE-A) systemic lupus erythematosus (A), with (LN+) or without (no LN) lupus nephritis (B) and control group (NC). ANOVA, T-Student. Significance: $p < 0.05$.

CONCLUSION

The present study sought to show phenotypic differences between active and inactive forms of SLE. It was demonstrated a reduction in T CD4, regulatory T and B cells compared to control, as well in NKt cells in patients with active form of the disease. By other hand, an increase of T CD8 cells was observed compared to healthy individuals. Data obtained in this study suggest a dysregulation in the immune response in patients with SLE and the occurrence of phenotypic alterations according to the disease status.

Although many questions related to the pathogenesis of SLE remain unanswered, it is possible that the progression of the disease results from a disruption in Treg cell-dependent peripheral self-tolerance. Our findings concerning regulatory T cells in patients with the active form of the disease indicate this loss of tolerance.

Among the parameters investigated in the present study, NKt cells were the only parameter significantly different between the active and inactive forms of SLE, suggesting that evaluation of these cells may play an important role in monitoring this complex disease. Therefore, these preliminary findings should guide the design of new studies to examine the relationship between subpopulations of NKt cells and disease activity.

This study may contribute to a greater knowledge about the pathogenesis of SLE in our population confirming some changes in the immune system of these patients. These changes are important in guiding strategies to control and develop new forms of treatment that may eventually provide a more effective response to the target population. New tools that predict the worsening of the disease also deserve to be investigated and are of great importance in adopting early actions in SLE.

CONFLICT OF INTEREST:

The authors of this study declare no relevant conflicts of interest.

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AUTHORS' CONTRIBUTIONS:

CMGL - conducted the experiments and drafted the manuscript; TMPBG - handwriting the manuscript; KSF - collected and processed the samples; MVSF - collected and processed the samples; LCVA - collected and processed the samples and manuscript submission; WBC - collected and processed the samples; FMFC - reviewed the cytometry analyzes; RVC - evaluation and recruitment of participants; CLSN - evaluation and recruitment of participants; PMP - evaluation and recruitment of participants; LIS - assisted in the standardization of antibodies; JDM - assisted in the statistical analysis; VPCPT - purchase of reagents and handwriting; MGC - Contribution to the writing of the manuscript and purchase of reagents.

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