

# Lymphocytotoxic antibodies in systemic lupus erythematosus are associated with disease activity irrespective of the presence of neuropsychiatric manifestations

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**Objectives:** To evaluate the association of the presence of lymphocytotoxic, anti- $\beta$ 2-glycoprotein I (anti- $\beta$ 2-GPI) and anti-ribosomal P (anti-P) antibodies in patients with systemic lupus erythematosus (SLE), presenting or not neuropsychiatric (NP) manifestations, stratified according to the activity of the disease.

**Methods:** A total of 138 patients with SLE (59 with active NPSLE, 49 with active non-NPSLE, and 30 with inactive disease) and 57 healthy controls were studied. Disease activity was assessed by the SLE Disease Activity Index (SLEDAI). The presence of lymphocytotoxic antibodies was assessed using a complement-dependent lymphocytotoxicity assay. The presence of anti- $\beta$ 2-GPI and anti-P antibodies was detected by enzyme-linked immunosorbent assay (ELISA).

**Results:** Lymphocytotoxic antibodies were detected primarily in patients with active disease, that is in 35 out of 59 (59.3%) NPSLE and 23 out of 49 (46.9%) non-NPSLE patients, whereas only four out of 30 (13.3%) inactive SLE patients and none of the healthy controls exhibited the autoantibody. The frequency of lymphocytotoxic antibodies in active SLE patients, considered as a whole or stratified into NPSLE or non-NPSLE, was significantly increased in relation to inactive SLE patients ( $p < 0.001$  for each comparison). No significant difference was observed when comparing active NPSLE with non-NPSLE patients. No associations were observed between the presence of anti- $\beta$ 2-GPI or anti-P antibodies and the activity of SLE or the presence of lymphocytotoxic antibodies.

**Conclusions:** Lymphocytotoxic antibodies occurred more frequently in patients with active SLE than in patients with inactive disease, irrespective of the presence of NP manifestations, a finding that is similar to classical biomarkers of lupus activity (anti-dsDNA and complement). These results indicate that the assessment of the presence of lymphocytotoxic antibodies may be an additional useful tool for the evaluation of SLE activity.

Systemic lupus erythematosus (SLE) involves the most extensive clinical and serological diversity among the autoimmune diseases. The spectrum of clinical manifestations in SLE patients is broad, varying from subtle symptoms to potentially lethal conditions, with irreversible organ damage. Clinical management of SLE therefore requires reliable and specific biochemical markers to assess the disease activity and response to treatment (1–3).

Nervous system involvement occurs in up to 70% of SLE patients at some time during the course of the illness (4, 5). The central and peripheral nervous systems, the autonomic system, and the myoneural junction are the major affected areas (6). The

heterogeneity of the clinical presentations makes a definitive diagnosis of neuropsychiatric SLE (NPSLE) very difficult, as no ‘gold standard’ marker has been available for such investigation, and the diagnosis has been based primarily on the clinical picture (7, 8). Nevertheless, lymphocytotoxic, anti-phospholipid, and anti-P ribosomal antibodies have been associated with the presence of NP abnormalities in SLE (9–11).

Although lymphocytotoxic antibodies may occur in infectious, malignant, inflammatory bowel and autoimmune diseases, these antibodies have been reported to cross-react with neurons (12), and cognitive dysfunctions have been more consistently associated with the presence of lymphocytotoxic antibodies in SLE patients (13–19). In addition, NP symptoms, cutaneous lesions, fever, and haematological disturbances are more commonly seen in SLE patients presenting with lymphocytotoxic antibodies (12–15). As the positivity of lymphocytotoxic antibodies is similar in SLE patients exhibiting or not NP

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Accepted 27 May 2007

manifestations, the specificity of such antibodies for brain tissue has been questioned (19–21).

Antibodies against phospholipid components are a heterogeneous group of proteins, primarily associated with a noninflammatory vasculopathy, producing focal ischaemic neurological events, such as stroke, transverse myelitis, and amaurosis fugax, or diffuse neurological features including seizures, cognitive dysfunction, and psychosis (10, 22). The cationic plasmatic  $\beta$ 2-glycoprotein I ( $\beta$ 2-GPI) is a major cofactor involved in the binding of antibodies to anionic phospholipid-covered surfaces, and the expression of  $\beta$ 2-GPI occurs in various cell types, including lymphocytes of SLE patients. These findings suggest that antibodies against  $\beta$ 2-GPI may act as lymphocytotoxic antibodies participating in the pathogenesis of the NP manifestations of SLE (23).

Autoantibodies to ribosomal P proteins in SLE patients have been recognized for almost two decades (24), being highly prevalent in patients with SLE presenting with psychosis (11). As the immunoglobulin G (IgG) anti-ribosomal P (anti-P) antibodies found in SLE serum can bind to a 38-kDa molecule present on human lymphocytes (25), these antibodies may form a subgroup of lymphocytotoxic antibodies (26).

As lymphocytotoxic, anti- $\beta$ 2-GPI, and anti-P autoantibodies have been associated with the presence of NP manifestations in SLE patients, and  $\beta$ 2 GP and ribosomal P proteins are expressed on lymphocyte surfaces, antibodies against these proteins may in fact be lymphocytotoxic antibodies. To address the question of whether these antibodies could be used as markers of disease activity, in this study we investigated the presence lymphocytotoxic, anti- $\beta$ 2-GPI, and anti-P autoantibodies in serum of SLE patients exhibiting or not NP manifestations, stratified according to clinical (active versus inactive disease) and classic laboratory variables associated with disease activity (peripheral blood lymphocyte

number, anti-dsDNA antibody, and complement levels).

## Patients and methods

### Patients

We studied 138 patients (seven men) aged 15 to 56 years (mean age 36.4 years) with SLE, diagnosed according to the criteria of the American College of Rheumatology (ACR) (27) and seen at the University Hospital of the School of Medicine of Ribeirão Preto, Brazil, from 1996 to 2005. NPSLE was defined according to the ACR standard nomenclature and case definition (28). Among the 59 patients with active NP features, 27 had seizures, 15 psychosis, 16 severe depression, 18 cerebrovascular disease, five acute confusional state, two myelopathy, three severe anxiety, and one aseptic meningitis. Some patients presented more than one NP manifestation. Patients who exhibited only headache were not included as NPSLE patients.

One hundred and eight patients exhibited active disease (59 with and 49 without NP manifestations), and 30 patients with inactive disease (eight with previous NP features and 22 non-NPSLE). Disease activity was measured by the SLE Disease Activity Index or SLEDAI-2000 (29, 30), considered active when  $\geq 2$ , and inactive when  $< 2$ . Blood samples of patients with active disease were collected before the institution of specific treatment. The demographic, clinical, and laboratory features of all patients are presented in Table 1.

### Controls

A total of 57 healthy blood donors without personal or family history of autoimmune diseases from the

Table 1. Demographic, clinical, and laboratory features of patients (n=138) in groups I (active NPSLE), II (active non-NPSLE), and III (inactive SLE).

	Active NPSLE (n=59)	Active non-NPSLE (n=49)	Inactive SLE (n=30)
Female, n (%)	56 (95)	46 (94)	29 (97)
Age, mean $\pm$ SD years	35.05 $\pm$ 13.9	36.3 $\pm$ 11.52	39.1 $\pm$ 11.18
SLEDAI, mean $\pm$ SD	18.5 $\pm$ 10.5	8.6 $\pm$ 6.1	0.03 $\pm$ 0.2
Lymphocytes, mean $\pm$ SD	1229.5 $\pm$ 896.6	1135.6 $\pm$ 672.3	1444.8 $\pm$ 724.2
Active neuropsychiatric involvement, n (%)	59 (100)	0	0
Active nephritis	29 (49)	22 (45)	0
Active arthritis	5 (8)	10 (20)	0
Active cutaneous	9 (15)	13 (26)	0
Active haematology	11 (19)	10 (20)	1 (0.03)
Active serositis	11 (19)	10 (20)	0
Auto-crossmatch positive, n (%)	35 (59.3)	23 (46.9)	4 (13.3)
Anti-dsDNA positive and/or low complement, n (%)	31 (52.5)	21 (42.8)	0
Anti- $\beta$ 2-GPI positive, n (%)	47 (79.6)	38 (77.5)	18 (60)
Anti-ribosomal P positive, n (%)	10 (17.6)	14 (33.3)	5 (16.7)

SLE, systemic lupus erythematosus; NPSLE, neuropsychiatric SLE; SLEDAI, SLE Disease Activity Index; GPI, glycoprotein I.

same geographic area as the patients were also assayed for lymphocytotoxic autoantibodies.

All subjects gave written informed consent to participate in the investigation, and the study protocol was approved by the Ethics Committee of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil.

## Methods

### Lymphocytotoxic antibodies

Lymphocytes were separated from freshly drawn peripheral blood cells by gradient density centrifugation using Fycoll-Hypaque (density 1.077) Cederline (Hornby, Ontario, Canada). Serum was collected simultaneously from each patient at the time of clinical evaluation. Lymphocytotoxicity determination was performed using a classical complement-dependent assay (20, 31) with minor modifications. A total of  $3 \times 10^6$  lymphocytes were incubated with 1  $\mu$ L of undiluted and serial diluted (1:2 to 1:128) serum for 30 min at 22°C using a 60-well high-profile sterile Terasaki tray (Robbins Scientific, Sunnyvale, CA, USA). After the addition of 5  $\mu$ L of rabbit complement, the tray was further incubated for 45 min at 22°C. Undiluted foetal bovine serum was used as negative control, and multiparous serum or commercial anti-lymphocyte globulin was used as positive control. Lymphocyte death was detected using Trypan blue dye diluted 1:3, and the test was considered to be positive when at least 20% of cell lysis was observed.

### Other autoantibodies and complement levels

Anti- $\beta$ -2-GPI antibodies were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (INOVA QUANTA Lite™  $\beta$ 2 GPI Screen, San Diego, CA, USA). Anti-P antibodies were also determined by ELISA (INOVA QUANTA Lite™

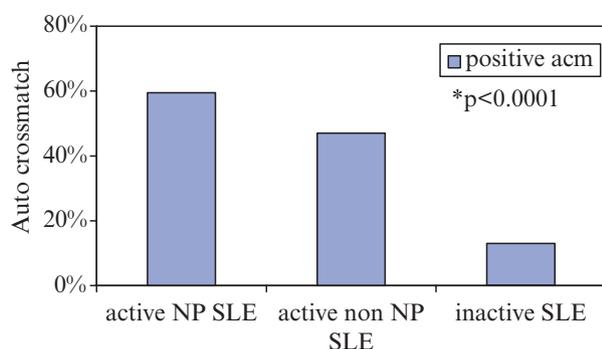


Figure 1. Frequencies of auto-crossmatch (ACM) in patients with active neuropsychiatric systemic lupus erythematosus (NPSLE) (n=59), in patients with active non-NPSLE (n=49), and in patients with inactive SLE (n=30).

Ribosome P, San Diego, CA, USA). Anti-dsDNA antibodies were detected using the *Crithidia luciliae* assay (INOVA Lite™ dsDNA *Crithidia luciliae*, San Diego, CA, USA). Serum levels of C3 and C4 were determined by nephelometry (Dade Behring Inc., Newark, DE, USA).

### Statistical analysis

A two-tailed Fisher's exact test was used for the associations regarding the autoantibody profile, while the Student t-test was used to compare the number of peripheral blood lymphocytes, both considered to be significant at  $p < 0.05$ .

## Results

Among patients with active NPSLE, 35 (59.3%) presented lymphocytotoxic, 47 (79.6%) anti- $\beta$ -2-GPI, and 10 (17.5%) anti-P antibodies. Twenty-three (46.9%) active non-NPSLE patients presented lymphocytotoxic, 38 (77.5%) anti- $\beta$ -2-GPI, and 14 (33.3%) anti-P antibodies. Among patients with inactive SLE, four (13.3%) exhibited lymphocytotoxic, 18 (60%) anti- $\beta$ -2-GPI, and five (16.7%) anti-P antibodies (Table 1).

Regarding the presence of lymphocytotoxic antibodies, no significant differences were observed when patients presenting active NPSLE were compared to those with active non-NPSLE; however, a significant difference was observed when patients with active disease (NPSLE plus non-NPS) were compared to those with inactive disease ( $p < 0.0001$ ). The correlation between SLEDAI and anti-lymphocytotoxic titres was not made because all patients with active disease presented anti-lymphocytotoxic antibodies titres higher than 1/128. No lymphocytotoxic antibodies were observed among healthy controls. These data suggest that the occurrence of lymphocytotoxic antibodies may be a marker of SLE activity (Figure 1). In addition, among active SLE patients, 52.54% of NPSLE and 42.8% of non-NPSLE presented anti-dsDNA antibodies or low levels of complement C3 and C4 components or both. All patients with inactive disease exhibited no anti-dsDNA antibodies and normal levels of C3 and C4 complement components. When active patients were considered together, 56 (51.8%) had anti-dsDNA antibody or low levels of complement components or both ( $p = 0.04$ ).

Irrespective of the presence or not of NP features, no association between the presence of anti- $\beta$ -2-GPI antibodies and SLE activity was observed. Similar behaviour was observed regarding the presence of anti-P antibodies (Table 2). When we evaluated the relationship between lymphocytotoxic autoantibodies and anti- $\beta$ -2-GPI or anti-P antibodies, no significant differences were observed (Table 3).

Table 2. Correlation between the positivity of anti- $\beta$ 2 glycoprotein I (GPI) or anti-ribosomal P (anti-P) antibodies and the subsets of clinical manifestations of SLE (neuropsychiatric or non-neuropsychiatric).

	Positive anti- $\beta$ 2 GPI	Negative anti- $\beta$ 2 GPI	p	Positive anti-P	Negative anti-P	p
Active SLE	85	23	0.056	24	74	ns
Inactive SLE	18	12		5	25	
Active NPSLE	47	12	0.07	10	47	ns
Inactive SLE	18	12		5	25	
Active non-NPSLE	38	11	ns	14	27	ns
Inactive SLE	18	12		5	25	
Active NPSLE	47	12	ns	10	47	ns
Active non-NPSLE	38	11		14	27	

SLE, systemic lupus erythematosus; NPSLE, neuropsychiatric SLE; ns, not significant.

Lymphopaenia was observed in the three groups of patients studied in the present series, being observed in 76.3% of active NPSLE patients, 79.6% of active non-NPSLE patients, and 60% of inactive SLE patients (see Table 1); however, no significant differences were observed when these groups were compared. The comparisons of the number of peripheral blood lymphocytes between patients presenting or not lymphocytotoxic antibodies showed no significant differences (data not shown).

## Discussion

As lymphocytotoxic, anti- $\beta$ 2-GPI, and anti-P antibodies have been associated with the development of NP manifestations of SLE, this study was designed to evaluate the associations between the presence of these autoantibodies in SLE patients exhibiting or not NP disorders and stratified according to disease activity.

Using the auto-crossmatch (ACM) method, we were able to detect the presence of lymphocytotoxic antibodies in SLE patients with active disease, irrespective of the presence or not of NP manifestations, whereas SLE patients with inactive disease

exhibited no such antibodies. Some authors have argued that lymphocytotoxic autoantibodies may cross-react with brain structures and may be responsible for NP manifestations of SLE (13, 14, 16, 18), while others have not found such association (19–21). As the frequency of these lymphocytotoxic antibodies was very similar in active SLE patients of the present study, these findings seem to be related to the activity of the disease rather than to the NP features (19, 20). This is an interesting finding, considering the scarcity of reliable biomarkers associated with SLE activity. Anti-dsDNA antibodies (32–34), the quantification of C3 and C4 components of complement (32), the evaluation of the total haemolytic complement activity (CH50) (32), and the widely used SLEDAI score (35), have been useful laboratory tools to assess disease activity. In our study, the two groups of patients with active disease (assessed by SLEDAI-2000) exhibited higher frequency of lymphocytotoxic autoantibodies than anti-dsDNA antibodies and/or low levels of C3/C4 complement components. Recently, new biomarkers have been proposed in an attempt to determine SLE activity, such as erythrocyte-bound complement activation product C4d (36), CD27<sup>high</sup> plasma cells (37), anti-nucleosome antibodies (38–40), anti-C1q antibodies (41–43), and anti-C-reactive protein antibodies (44). Our results suggest that the assessment of the presence of lymphocytotoxic antibodies by the ACM method may be an additional useful tool of low cost and easy application for the determination of SLE activity.

Although the ACM identified lymphocytotoxic antibodies, lymphopaenia occurred independently of ACM positivity. This is an unexpected finding if we consider that the ACM indicates the presence of lymphocytotoxic antibodies, and consequently lymphopaenia should be more intense in patients with a positive ACM. In addition, no significant differences were observed regarding the peripheral blood lymphocytes in the three groups studied. The major reason for these findings may be related to the observation that lymphocytotoxic antibodies are primarily directed against naïve lymphocytes (45), that is they target only a subset of lymphocytes.

Table 3. Correlation between positivity of auto-crossmatch (ACM) and the presence of anti- $\beta$ 2 glycoprotein I (GPI) or anti-ribosomal P antibodies (anti-P) in groups I (active NPSLE), II (active non-NPSLE), and III (inactive SLE).

	Active NPSLE			Active non-NPSLE			Inactive SLE		
	ACM +	ACM-	p	ACM+	ACM-	p	ACM+	ACM-	p
Anti- $\beta$ 2 GPI positive	28	19	ns	18	20	ns	3	15	ns
Anti- $\beta$ 2 GPI negative	7	5		5	6		1	11	
Anti-P positive	7	3	ns	6	8	ns	1	4	ns
Anti-P negative	27	20		11	16		3	22	

SLE, systemic lupus erythematosus; NPSLE, neuropsychiatric SLE; ns, not significant.

Besides lymphocyte destruction by lymphocytotoxic antibodies, it should be emphasized that another major mechanism of lymphocyte destruction is increased apoptosis, with the observation of an inverse correlation between the lymphocyte apoptosis rate and the number of peripheral blood lymphocytes, particularly in NPSLE patients (46).

In this study, no significant associations were observed between the presence of anti- $\beta$ 2-GPI antibodies with disease activity or with the occurrence of NP features. In addition, no association was found between the presence of lymphocytotoxic antibodies and anti- $\beta$ 2-GP. Although  $\beta$ 2-GP is expressed on the surface of lymphocytes (23), the findings of the present study suggest that the antigen recognized by lymphocytotoxic antibodies is distinct. Similarly, no association between the presence of lymphocytotoxic and anti-P antibodies was observed in SLE patients presenting with active or inactive disease. The only association with this antibody was with psychiatric manifestations (28.6%) compared with those presenting with neurological symptoms only (6.7%), a finding previously reported by many other groups (11, 47–51).

In conclusion, the results presented here indicate that the ACM method to detect lymphocytotoxic antibodies, together with classic markers of lupus activity (anti-dsDNA and complement), is a useful tool for assessing SLE activity.

### Acknowledgements

This study was supported by the following Brazilian Research Foundations: Fundação de Apoio ao Ensino, Pesquisa e Assistência do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto – USP (FAEPA), and Fundação Hemocentro da Faculdade de Medicina de Ribeirão Preto – USP.

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