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CURSO DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA EM SAÚDE E MEDICINA INVESTIGATIVA

DISSERTAÇÃO DE MESTRADO

AVALIAÇÃO DO POLIMORFISMO GENÉTICO DE TLR9 EM PACIENTES COM LÚPUS ERITEMATOSO SISTÊMICO

LÚCIO MACEDO BARBOSA

Salvador – Bahia – Brasil
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Dissertação submetida à coordenação da Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa, Fundação Oswaldo Cruz, para a obtenção do Título de Mestre.

Orientador: Dr. Mitermayer Galvão dos Reis
Co-Orientador: Dr. Mittermayer Barreto Santiago

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"AVALIAÇÃO DO POLIMORFISMO GENÉTICO DE TLR9 EM PACIENTES COM LÚPUS ERITEMATOSO SISTÊMICO"

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Aos meus pais, pelo afeto, carinho, dedicação, e conselhos;

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Resumo

INTRODUÇÃO: Lúpus Eritematoso Sistêmico (LES) é uma doença autoimune sistêmica na qual a principal alteração está relacionada à ativação policlonal de linfócitos B com a produção de uma ampla variedade de autoanticorpos dirigidos contra componentes nucleares, citoplasmáticos, de superfície celular e moléculas solúveis do plasma. Fatores genéticos têm sido implicados na patogênese dessa patologia. Toll-like receptor 9 (TLR9) é um importante componente do sistema imune inato que reconhece sequências não metiladas CpG de DNA e a sua presença foi associada com o desenvolvimento de anticorpos anti-DNA dupla fita. OBJETIVOS: Investigar a prevalência de mutações pontuais (SNPs) (-1237 C>T, +1174 A>G, +2848 G>A) em um grupo de pacientes com LES brasileiros e estudar a sua associação com manifestações clínicas da doença. MÉTODOS: 158 pacientes diagnosticados com LES, com base nos critérios do ACR (1997), foram incluídos no estudo. Todos os SNPs foram identificados através de sequenciamento de fragmentos no gene do TLR9. As sequências foram analisadas usando o software SeqScape® 5.1. Esse estudo foi aprovado pelo comitê de ética de nossa instituição. RESULTADOS: A frequência genotípica encontrada na população estudada foi de 56,3, 37,3 e 6,3% para TT, CT e CC, respectivamente, na posição -1237, 38,6, 41,1, e 20,3% para GG, GA e AA, respectivamente, para a posição +1174, e 23,4, 45,6 e 31,0% para AA, AG e GG, respectivamente, para a posição +2848. A frequência alélica na posição -1237 foi de 74,95 e 24,25% para T e C, respectivamente, na posição +1174 foi de 59,15 e 40,85%, respectivamente para G e A e na posição +2848 foi de 46,2 e 53,8% para A e G, respectivamente. As frequências genotípicas de todos os SNPs se apresentaram em equilíbrio de Hardy-Weinberg. Avaliando a associação entre polimorfismos e sinais clínicos foi encontrada uma correlação entre a homozigose do alelo C na posição -1237 e psicose ($p<0,01$). Da mesma forma, homozigose do alelo G na posição +2848 foi associada com eritema discóide. CONCLUSÃO: Esses dados mostram que SNPs no gene do TLR9 podem estar associados com o desenvolvimento de sintomas clínicos específicos em pacientes com LES.

Palavras Chave: Lúpus Eritematoso Sistêmico; Toll-like receptor 9; polimorfismos; psicose

Abstract

INTRODUCTION: Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disease secondary to a failure of the immune system leading to a polyclonal activation of B cells with the production of a broad range of autoantibodies directed against nuclear and cytoplasmic components, and plasmic soluble molecules. Genetic background has been implicated as contributing factor for its development. Toll-like receptor 9 (TLR9) is an important component of the innate immune system that recognizes unmethylated CpG-DNA and its presence has been associated with the development of anti-dsDNA autoantibodies in a murine model of SLE. OBJECTIVE: To investigate the prevalence of TLR9 single nucleotide polymorphisms (SNPs) (-1237 C>T, +1174 A>G, +2848 G>A) in a group of Brazilian SLE patients and to study its association with clinical manifestation of the disease. METHODS: An unselected group of a hundred and fifty eight patients with SLE based on the American College of Rheumatology criteria was included in the study. All SNPs were identified by sequencing fragments of the TLR9 gene. The sequences were analyzed using SeqScape® 5.1 software. The study was approved by the Ethics Committee of our institution. RESULTS: The overall genotype frequencies in the studied population were 56.3, 37.3, and 6.3% for TT, CT and CC respectively, at position -1237; 38.6, 41.1 and 20.3% for GG, GA and AA, respectively, at position +1174; and 23.4, 45.6 and 31.0% for AA, AG and GG, respectively, at position +2848. The allele frequencies at position -1237 were 74.9 and 24.1% for T and C, respectively, at position +1174 they were 59.2 and 40.8% for G and A, respectively, at position +2848 they were 46.2 and 53.8% for A and G, respectively. Frequency of genotype distributions in all SNPs met Hardy–Weinberg's expectation. There was an association between homozygosis of allele C at position -1237 and psychosis ($p<0.01$). Likewise, homozygosis of allele G at position +2848 was associated with discoid rash ($p<0.05$). CONCLUSION: These data show that TLR9 SNPs may be associated with the development of specific clinical symptoms in patients with SLE.

Keywords: Systemic lupus erythematosus; toll-like receptor 9; polymorphism; psychosis

LISTA DE ABREVIATURAS

ACR: *American College of Rheumatology* (Colégio Americano de Reumatologia)

Anti-Sm: Anti-Smith

Anti-dsDNA: Anti-DNA dupla fita

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

CpG-ODN: CpG-Oligodesoxinucleotídeo

DNA: Ácido Desoxirribonucléico

EUA: Estados Unidos da America

HLA: *Human Leukocyte Antigen* (Antígeno Leucocitário Humano)

IL10: Interleucina 10

kb: Kilobases

LES: Lúpus Eritematoso Sistêmico

LPS: Lipopolissacarídeos

PAMPs: *Pathogens Associated Molecular Patterns* (Padrões Moleculares Associados a Patógenos)

RNA: Ácido Ribonucleico

RNAm: RNA mensageiro

RNAt: RNA transportador

snRNA: *Small Nuclear* RNA

SLE: *Systemic Lupus Erithematosus* (Lúpus Eritematoso Sistêmico)

SNPs: *Single Nucleotide Polymorphisms* (Polimorfismos de Único Nucleotídeo)

SPSS: Statistical Package for the Social Sciences

TLR: Toll-like Receptor

TNF: *Tumor Necrosis Factor* (Fator de Necrose Tumoral)

VDRL: *Venereal Disease Research Laboratory* (Pesquisa Laboratorial de Doenças Venéreas)

SUMÁRIO

1. Introdução

O lúpus eritematoso sistêmico (LES) é o protótipo de uma doença multissistêmica de origem autoimune cuja principal característica imunológica é a ativação policlonal de linfócitos B com a produção de uma ampla variedade de auto-anticorpos dirigidos contra componentes nucleares, citoplasmáticos, de superfície celular e moléculas solúveis do plasma (MOK *et al.* 2003). As manifestações clínicas do LES variam de quadros discretos de artrite e eritema cutâneo a condições potencialmente fatais como o grave comprometimento do sistema nervoso central, renal, cardíaco e pulmonar, ou seja, virtualmente qualquer órgão ou sistema pode estar envolvido na evolução da doença (ABBAS, 2005).

Devido à grande variabilidade nas manifestações clínicas o *American College of Rheumatology* (Associação Americana de Reumatologia) estabeleceu uma lista de onze critérios de classificação para o LES. A definição de LES requer o preenchimento de quatro ou mais dos critérios a seguir: 1) eritema malar; 2) fotossensibilidade; 3) eritema discóide, 4) úlcera oral, 5) artrite, 6) serosite, 7) doença renal (avaliada pela presença de proteinúria ou cilindrúria), 8) doença neurológica (convulsões ou psicose sem outra causa aparente), 9) doença hematológica (anemia hemolítica, leucopenia ou trombocitopenia), 10) Alteração imunológica [auto-anticorpos anti-DNA de dupla fita (anti-dsDNA), anti-Sm ou antifosfolípides] e 11) anticorpos antinucleares (EGNER, 2000).

A complexidade do diagnóstico e a dificuldade dos pacientes em procurar um reumatologista, devido à grande variedade dos sintomas, tornam difícil estimar a taxa exata de incidência do LES. O que se sabe realmente é que o LES afeta mais mulheres que homens, em uma proporção de 7-15:1 (LAHITA, 1999), e que há uma maior prevalência na população afro-descendente (MANZI, 2001). Em um estudo realizado no sudeste da China, Mok *et al.* encontraram no período de 2000 a 2006 uma taxa de incidência anual de 3,1/100.000 habitantes sendo 5,4/100.000 mulheres (MOK *et al.* 2008). No Reino Unido, no período de 1990 a 1999, foi descrito uma taxa de incidência anual de 7,89/100.000 mulheres e 1,53/100.000 homens (SOMERS *et al.* 2007). No nordeste do Brasil, em Natal, a taxa de incidência encontrada foi de 8,7/100.000 habitantes/ano sendo 14,1/100.000 mulheres e 2,2/100.000 homens (VILAR & SATO, 2002). Em geral, acredita-se que a taxa de incidência anual do LES seja de 1 por 2500 indivíduos da população geral e 1 por 700 mulheres em idade fértil. A prevalência de casos (por 100.000 indivíduos) varia entre diferentes etnias e é estimada em 03-19 para homens caucasianos, 03-53 entre homens afro-americanos, 17-71 entre mulheres caucasianas e 56-283 em mulheres afro-americanas (MANZI, 2001).

Acredita-se que a maior freqüência do LES no sexo feminino acontece devido a ação do hormônio feminino estrógeno. Isso pode ser evidenciado pela diminuição da razão entre a proporção entre os gêneros em crianças, período no qual os efeitos dos hormônios sexuais não são tão importantes, a qual se torna de três meninas para cada menino (LAHITA, 1999). Outros pontos que apóiam essa hipótese são as maiores taxas de risco de desenvolver LES em pacientes que tiveram menarca precoce ou que fazem algum tipo de tratamento baseado em estrógeno, mulheres utilizando contraceptivos

orais ou que fazem reposição hormonal (COSTENBADER *et al.* 2007). Enquanto os hormônios sexuais femininos demonstram ser um fator de risco para o desenvolvimento ao LES, a testosterona, hormônio sexual masculino, demonstra se correlacionar inversamente com a atividade de doença. Foi demonstrado que pacientes do sexo masculino e com LES apresentam baixas concentrações de testosterona e altas taxas de hormônio luteinizante comparados aos pacientes saudáveis (SEQUEIRA *et al.* 1993; MOK *et al.* 2000; LAHITA *et al.* 1987), demonstrando assim que taxas excessivas de hormônios sexuais femininos e taxas de hormônios andrógenos anormais podem ser responsáveis por alterações no sistema imune.

Apesar das evidências mostrarem a importância dos hormônios sexuais no surgimento do LES, a etiologia desta doença ainda não está completamente elucidada. Postula-se que para o surgimento do LES deve haver uma associação entre os fatores genéticos do indivíduo e os fatores ambientais que o cercam (RAHMAN & ISENBERG, 2008). Os fatores ambientais se mostraram importantes quando foram identificadas algumas drogas que causam indistinguíveis do LES idiopático, como a hidralazina e procainamida (RUBIN, 2002). Este tópico é reforçado quando se observa a influência da luz ultravioleta na exacerbação dos sintomas do LES (HOCHBERG, 1997). A predisposição genética para o LES é comprovada principalmente pela ocorrência da patologia em mais de um membro da mesma família e pela maior taxa de concordância da doença em gêmeos monozigóticos, 25-70%, quando comparados a dizigóticos, 2-9% (GRENNAN *et al.* 2007). Apesar desses dados, é estimado que pelo menos quatro genes sejam necessários para o surgimento do LES (SCHUR, 1995). Desta forma, muitos são os trabalhos que tentam realizar uma associação genética com o LES. Os

genes mais estudados são os HLA de classe II, mostrando inclusive uma associação destes com a produção de autoanticorpos específicos, como anti-dsDNA, anti-Sm (antígeno Smith, que é uma ribonucleoproteína) e anti-fosfolípides (RAHMAN & ISENBERG, 2008).

A presença de autoanticorpos é uma das principais características do LES. Anticorpos direcionados contra DNA, RNA e proteínas associadas representam uma peça chave na patogênese do LES, contudo o estímulo para a formação dos mesmos e o exato papel no surgimento dos sintomas não é completamente esclarecido (LAFYATIS e MARSHAK-ROTHSTEIN, 2007). Devido a estes fatos, muitas moléculas, como os próprios anticorpos, e células, como linfócitos B e T autorreativos, envolvidas no sistema imune adaptativo são amplamente estudados. O sistema imune inato passou a ser mais estudado na patogenia de doenças autoimunes, baseado no fato de que a grande maioria dos modelos murinos experimentais necessitam de adjuvantes para iniciar a resposta imunológica, que frequentemente são estímulos da imunidade inata, como LPS, flagelina, CpG-ODN, entre outros. (LAFYATIS e MARSHAK-ROTHSTEIN, 2007).

As principais moléculas que envolvem o sistema imune inato são os receptores que reconhecem padrões moleculares. Antigamente acreditava-se que esses receptores reconheciam apenas padrões moleculares associados a patógenos (PAMPs), mas com o avançar das pesquisas descobriu-se que esses receptores também reconhecem padrões moleculares do hospedeiro (AKIRA S, UEMATSU S e TAKEUCHI O, 2006). Como o sistema imune inato é menos eficiente em distinguir o próprio do não-próprio,

quando comparado ao sistema imune adaptativo, acredita-se que esse reconhecimento seja a chave do surgimento de doenças autoimunes (LAFYATIS e MARSHAK-ROTHSTEIN, 2007).

Os *toll-like receptors* (TLR) constituem a mais importante família de receptores que reconhecem padrões moleculares envolvidas no LES. Esta família possui 13 tipos distintos de receptores, diferenciados por números, que são capazes de reconhecer diferentes componentes microbianos, sejam eles virais ou bacterianos. (NAPOLITANI *et al.* 2005; AKIRA S, UEMATSU S E TAKEUCHI O, 2006). O *toll-like receptor* 9 (TLR9) foi descrito como a molécula responsável pela maturação de células dendríticas e produção de citocinas pró-inflamatórias por macrófagos em resposta a reconhecimento de DNA não-metilado bacteriano em camundongos, desempenhando papel semelhante em humanos (HEMMI *et al.* 2000; BAUER *et al.* 2001).

Desta forma, baseando-se na importância dos receptores de reconhecimento de padrões moleculares, envolvidos nos mecanismos da imunidade inata, na patogênese do LES, pesquisadores na Inglaterra, em 2005, descreveram a primeira associação entre LES e o TLR9 em modelos experimentais de LES. Eles postularam que a formação de anticorpos anti-dsDNA e anti-cromatina são dependentes de TLR9, mas que a falta desse gene, conseqüentemente anti-dsDNA, não tem efeito no desenvolvimento da doença ou da nefrite, tendo em vista que estes auto-anticorpos são reconhecidamente associados com o envolvimento renal no LES (CHRISTENSEN *et al.* 2005).

O gene do TLR9 está localizado no cromossomo 3p21.3, possui aproximadamente 5 kb e é constituído de dois exons, sendo que a maior região codificadora encontra-se no segundo exon (DU et al. 2000; KELLY et al. 2002). Apesar do gene do TLR9 ser bem conservado entre as espécies, pequenas diferenças nas sequências gênicas, ou seja, mutações, poderiam estar envolvidas no desenvolvimento de doenças humanas associadas a respostas alteradas do sistema imune inato, como asma, trombose venosa profunda, e doença pulmonar obstrutiva crônica (BAUER *et al.* 2001). Desta forma pesquisadores dos EUA realizaram um estudo a fim de descrever o gene do TLR9. Eles estudaram 71 pacientes de diferentes etnias (afro-americanos, hispânicos e caucasianos) e encontraram 20 polimorfismos pontuais (SNPs), destes apenas quatro estavam presentes em todas as diferentes populações estudadas. Dois destes SNPs encontram-se nas na região promotora do gene, o da posição -1486 onde há uma substituição de uma timina por uma citosina (-1486 T>C) e o da posição -1237, onde há uma transição de uma citosina por uma timina (-1237 C>T), outro polimorfismo encontra-se no intron, na posição +1174, onde há uma substituição de uma adenina por uma guanina (+1174 A>G), e o último SNP encontra-se no segundo exon, na posição +2848, que é uma transição, que causa uma mutação silenciosa, de uma guanina por uma adenina (+2848 G>A) (LAZARUS *et al.* 2003).

Os estudos realizados por Lazarus *et al.* (2003), que descreve os polimorfismos no gene do TLR9 e Christensen *et al.* (2005), que relata a primeira associação entre o gene do TLR9 e o surgimento de anticorpos anti-dsDNA em modelos experimentais de LES, despertaram o interesse de outros grupos realizar pesquisas procurando associar o polimorfismo genético do TLR9 em humanos com a susceptibilidade ao LES. No

entanto, os resultados na literatura demonstram ser controversos. Enquanto Tao *et al.* em 2007 e Xu *et al.* em 2009 descreveram a associação de polimorfismos do gene de TLR9, +1174 A>G e +2848 G>A respectivamente, com o risco de desenvolver LES em uma população japonesa e chinesa, concomitantemente, outros estudos realizados em diferentes populações, relatam que tal associação não foi encontrada (TAO *et al.* 2007; XU *et al.* 2009; DEMIRCI *et al.* 2007; De JAGER *et al.* 2006; NG *et al.* 2005; HUR *et al.* 2005). Destes seis trabalhos publicados, apenas dois procuram avaliar a associação destes polimorfismos com o surgimento de sintomas do LES. Ng *et al.* (2005) acharam, sem significância estatística (p=0,056), um número elevado de pacientes com serosite e com o polimorfismo -1486 T>C (61,1% vs 44,1%) e Hur *et al.* em 2005 não descreveram associação alguma entre sintomas do LES com polimorfismos do TLR9.

No Brasil, e especialmente na Bahia, que possui características étnicas distintas das populações já estudadas, este tipo de trabalho não foi realizado. Desta forma, o objetivo deste estudo foi descrever a freqüência dos polimorfismos no gene do TLR9 em pacientes com LES residentes na Bahia e estudar uma associação com os diversos sintomas desta condição clínica.

2. Objetivo

2.1 Geral

Estudar a associação entre polimorfismos genéticos do TLR9 e Lúpus Eritematoso Sistêmico.

2.2 Específicos

- Descrever a freqüência dos polimorfismos -1237 C>T, +1174 A>G e +2848 G>A no gene do Toll-like receptor 9 em pacientes com lúpus eritematoso sistêmico.
- Pesquisar a associação entre os polimorfismos -1237 C>T, +1174 A>G e +2848 G>A no gene do Toll-like receptor 9 e complicações do lúpus eritematoso sistêmico.

3. Material e Métodos

3.1 Pacientes

Foram pesquisados 158 pacientes diagnosticados com LES atendidos no serviço de reumatologia do Hospital Santa Izabel, caracterizando assim uma amostra de conveniência. Foram incluídos os pacientes que preenchiam pelo menos quatro dos 11 critérios para o diagnóstico de LES, estabelecidos pelo ACR em 1997 REF, cuja idade era igual ou acima de 18 anos e que assinaram o termo de consentimento livre e esclarecido (TCLE). Após a assinatura do TCLE os pacientes foram entrevistados e tiveram seu prontuário revisado a fim de preencher o banco de dados com informações relativas à epidemiologia e à história clínica do mesmo.

O desenho de estudo escolhido para analisar a associação entre os polimorfismos no gene do TLR9 e os sintomas foi o de caso-controle. Tendo em vista que o LES ocasiona diferentes tipos de sintomas, os grupos casos e controles foram modificados em razão de cada sinal clínico avaliado. Como critério de inclusão para o grupo dos casos, foi avaliada a presença de determinado sintoma em algum momento da história clínica, e o grupo de controles foi determinado a partir da ausência desta manifestação clínica no curso da doença.

4. Manuscrito

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Toll-Like Receptor 9 Polymorphims in Brazilian Patients with Systemic Lupus Erythematosus

[Polimorfismos do Toll-like Receptor 9 em Pacientes com Lúpus Eritematoso Sistêmico Brasileiros]

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ABSTRACT

INTRODUCTION: Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disease secondary to a failure of the immune system. Genetic background has been implicated as contributing factor for its development. Toll-like receptor 9 (TLR9) is an important component of the innate immune system that recognizes unmethylated CpG-DNA and its presence has been associated with the development of anti-dsDNA autoantibodies in a murine model. OBJECTIVE: To investigate the prevalence of TLR9 single nucleotide polymorphisms (SNPs) (-1237 C>T, +1174 A>G, +2848 G>A) in a group of Brazilian SLE patients and its association with clinical manifestation of the disease METHODS: An unselected group of patients with SLE based on the ACR criteria was included in the study. All SNPs were identified by sequencing the TLR9 gene. The sequences were analyzed using SeqScape® 5.1 software. The study was approved by the Ethics Committee of our institution. RESULTS: The overall genotype frequencies in the studied population were 56.3, 37.3, and 6.3% for TT, CT and CC respectively, at position -1237, 38.6, 41.1 and 20.3% for GG, GA and AA, respectively, at position +1174, and 23.4, 45.6 and 31.0% for AA, AG and GG, respectively, at position +2848. The allele frequencies at position -1237 were 74.95 and 24.25% for T and C, respectively, at position +1174 they were 59.15 and 40.85% for G and A, respectively, at position +2848 they were 46.2 and 53.8% for A and G, respectively. Genotype distributions in all SNPs met Hardy–Weinberg's expectation. There was an association between homozygosis of allele C at position -1237 and psychosis ($p<0.01$). Likewise, homozygosis of allele G at position +2848 was associated with discoid rash ($p<0.05$). CONCLUSION: These data show that TLR9 SNPs may be associated with the development of specific clinical symptoms in patients with SLE.

Keywords: Systemic lupus erythematosus; toll-like receptor 9; polymorphism; psychosis.

INTRODUCTION:

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disease secondary to a loss of self-tolerance and production of autoantibodies against a wide range of different types of cellular and serum components[1]. Among the autoantibodies seen in SLE, anti-dsDNA have been implicated as a major source of nephritogenic stimuli[2]. Clinical manifestation in SLE varies from mild arthritis and cutaneous rashes to potentially fatal complications, involving central nervous system (CNS), kidney and blood vessels[3].

Toll-like receptor 9 (TLR9) is an important component of the innate immune system that recognizes unmethylated CpG-DNA, which is typical of prokaryotes. TLR9 gene is located on chromosome 3p21.3, encodes approximately 5 kb and has two exons, the second one being the major coding region[4,5]. Lazarus *et al.* in 2003 demonstrated 20 different SNPs in this gene, but only four were common in the three different ethnical groups, being two in the promoter region (-1486 T>C and -1237 C>T), one in the intron (+1174 A>G), one in the second exon (+2848 G>A), this one being a synonymous mutation[6].

The activity of TLR9 has been associated with the development of anti-dsDNA autoantibodies in a murine model[7]. The association between *TLR9* gene polymorphisms and SLE in humans is disputed. While Tao *et al.* (2007)[8], in a Japanese population, and Xu *et al.* (2009)[9], in a Chinese population, described associations between polymorphisms in *TLR9* gene and risk for the development of SLE, other studies performed in different populations failed to describe this association[10,11,12,13].

In the present study we investigated for the first time the prevalence of TLR9 single nucleotide polymorphisms (SNPs) (-1237 C>T, +1174 A>G, +2848 G>A) in a group of northeast Brazilian SLE patients and studied its association with clinical manifestation of this disease

MATERIAL AND METHODS:

Patients

A group of patients diagnosed with SLE based on the American College of Rheumatology criteria[14] who attended the Rheumatology Service of Hospital Santa Izabel in Salvador, Brazil was included in the present study. All invited patients agreed to participate in the study. The study was approved by The Ethics Research Committee of our institution and an informed consent was obtained from every patient prior to enrollment in the study.

Single Nucleotide Polymorphisms (-1237 C>T, +1174 A>G, +2848 G>A) of TLR9

Genomic DNA was extracted from peripheral blood mononuclear cells using 5 mL of whole blood using Qiagen DNA Blood Mini kit (Valencia – CA).

All SNPs were identified by amplifying fragments of the TLR9 gene by Polymerase Chain Reaction (PCR), and then sequencing these PCR products. Using primers described by Hur *et al* 2004, fragments of each SNP site were amplified by polymerase chain reaction (PCR). Each PCR mix had approximately 50 ng of DNA template, and 25 uM of each primer, 10 mM of DNTPs and 5 U of recombinant Taq DNA polymerase (Invitrogen). For better reaction specificity, temperature program was based on "Touch-Down" PCR variation. Thus, temperature program consisted of: 94º C for 3 minutes; 10 cycles of 3 steps: 94º C for 30 seconds, 65ºC for position -1237, 64º C for position +1174, 63.5º C for position +2848 for 30 seconds, on which temperature was reduced 0,5º C at every cycle until it reached the expected annealing temperature, and 72º C for 1 minute; 25 cycles of 94º C for 30 seconds, 60º C for position -1237, 59º C for position +1174, 58.5º C for position +2848 for 30 seconds and 72º C for 1 minute; and the final step of 72º C for 7 minutes.

PCR products were then purified with spin column PCR purification kit (Qiagen, Valencia – CA) and submitted to an electrophoresis agarose gel 2% with a low mass DNA ladder (Invitrogen) in order to determine DNA concentration for sequencing.

PCR product was sequenced in both directions, using sense and anti-sense primers (2 uM) using

Kit ABI Prism BigDye Terminator (Applied Biosystems-Hitachi), following manufactorer's instructions, and 3100 Genetic Analyser (Applied Biosystems-Hitachi).

Results were analyzed using bioinformatics software SeqScape® 5.1 (Applied Biosystems-Hitachi), comparing obtained DNA sequences with reference sequence of TLR9 gene published as NC 000003, in NCBI website.

With all the results Hardy-Weinberg equilibrium and Linkage disquilibrium were analyzed with GenePop[15].

Statistical analysis was performed using SPSS© 17.0 program (Chicago, Illinois). Variables that showed normal distribution, results were expressed as mean ± standard deviation and the variables showing unsymmetrical distribution, results were expressed as median with quartile 25 and 75. The association between qualitative variables was evaluated by chi-square corrected (Yates) or Fisher exact test, when indicated, considering $p < 0.05$ as statistically significant.

RESULTS

This study enrolled 158 patients with SLE. The overall genotype frequencies in the studied population were 56.3, 37.3, and 6.3% for TT, CT and CC, respectively, at position -1237, 38.6, 41.1 and 20.3% for GG, GA and AA, respectively, at position +1174, and 23.4, 45.6 and 31.0% for AA, AG and GG, respectively, at position +2848 (Table 1). The allele frequencies at position -1237 were 74.95 and 24.25% for T and C, respectively, at position +1174 they were 59.15 and 40.85% for G and A, respectively, at position +2848 they were 46.2 and 53.8% for A and G, respectively. The most frequent diplotypes seen in our population are displayed in Table2. Diplotypes with TT, GA and AG at positions -1237, +1174 and +2848, respectively, were present in 17.7% of the patients. The second diplotype more frequent showed in 10.8% individuals, had TT, AA and GG, at positions -1237, +1174 and +2848, respectively (Table 2). However, any of the diplotypes seen in our population correlated with any of the clinical symptoms. Genotype distributions in all

SNPs met Hardy–Weinberg's expectation. Putting our data through a linkage disequilibrium test, we could see that alleles at positions -1237 and +1174, and at positions +1174 and +2848 are seen in a frequency higher than expected.

The demographic data presented in table 3 shows that patients with and without mutations in the TLR9 gene were not different regarding to race, gender, age or disease duration.

Table 4 compares genotypes and clinical symptoms or complications. There was an association between homozygosis of allele C at position -1237 and CNS involvement ($p<0.05$), primarily psychosis ($p<0.01$) rather than seizures ($p=0.83$). Likewise, homozygosis of allele G at position +2848 was found to be associated with discoid rash ($p<0.05$). Table 5 demonstrates that when subgroups of patients were formed in order to compare clinical symptoms with genotypes, mean age remained similar, making groups comparables.

Analyzing disease duration with clinical symptom and genotype, we were able to see that patients with psychosis and homozygosis of allele C at position -1237 and discoid rash and homozygosis of allele G at position +2848 had an earlier clinical onset than patients with the same disease manifestation and other genotypes, even though no statistical significance was seen (Table 6).

DISCUSSION

SLE is an autoimmune disease which etiology remains unrevealed, but evidences show the importance of genetic factors for the development of the disease[16,17]. TLR9 recognizes unmethylated CpG-DNA sequences of prokaryotes, but it also recognizes endogenous DNA of SLE patients, making this molecule one of the potential explanations for lupus pathogenic onset[18]. Based on that hypothesis, researchers tried to identify whether genetic polymorphisms are associated with the lupus susceptibility, obtaining inconsistent results, even though they studied populations with different genetic backgrounds[5,6,7,8,9,10]. Since we did not include a

healthy Brazilian control group, this study was not able to verify if genetic polymorphisms of TLR9 is associated with SLE in an ethnical distinct group.

The aim of this study was to describe the prevalence of TLR9 polymorphisms in Brazilian SLE patients and to evaluate whether such genetic polymorphisms would be associated with any particular clinical manifestation or complication of the disease. We found that genotype CC at position -1237 is associated with CNS involvement ($p<0.05$), more precisely with psychosis ($p<0.01$), and that genotype GG at position +2848 is associated with discoid rash ($p<0.05$). From five previous studies involving genetic polymorphisms of TLR9 in SLE, Ng *et al.* (2005), Hur *et al.* (2005) and De Jager *et al.* (2006) were the only ones who tried to correlate this gene with clinical symptoms and could not find any association[8,9,10].

All of the SNPs evaluated in this study met Hardy-Weinberg expectations, as expected, but alleles at positions -1237 and +1174, and at positions +1174 and +2848 were seen in a frequency higher than expected. Since we did not perform a family-based association study we could not obtain the parental origin of each allele, making the concept of linkage disequilibrium not applicable. We believe the association between the before mentioned alleles is due to chance. However, since the SNPs we found associated with the clinical symptoms do not produce different forms of TLR9, the hypothesis of linkage disequilibrium with a non evaluated SNP as a cause of these findings should not be discarded.

A limitation of this study is that it was not population based, therefore, it may not be representative. The study population is composed of patients already attending in a tertiary rheumatologic unit and does not include any patients that are not undergoing treatment. Another potential limitation is the possibility that individuals with a "non mutant" genotype could go on to develop psychosis or discoid rash in the future.

Curiously, we observed that patients with psychosis who have mutations in position -1237 were younger and have shorter disease duration than patients with psychosis without mutation. Similarly, patients with discoid rash who have mutations in position +2848 were also younger and have shorter disease duration than patients with discoid rash without mutation (tables 5 and 6) perhaps implying that the reported SNPs are associated with early onset of such manifestation

In conclusion, TLR9 polymorphism is frequent in Brazilian SLE patients and SNPs at positions -1237 (C>T) and +2848 (G>A) are associated with CNS involvement and discoid rash. However, further studies are required to confirm such association in other populations with distinct genetic backgrounds and to explore the pathogenetic role of TLR9 gene on those clinical complications in SLE patients.

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Table 1 – Frequency of Genotypes in TLR9 gene in patients with SLE

Locus	Genotype	Total
-1237 C>T	TT/TC	148 (93,7)
	CC	10 (6,3)
+1174 A>G	GG/AG	126 (79,7)
	AA	32 (20,3)
+2848 G>A	AA/AG	109 (69,0)
	GG	49 (31,0)

Table 2 - Frequencies of Diplotypes in SLE patients

Diplotype	-1237 C>T	+1147 A>G	+2848 G>A	Frequency
Dip1	TT	GA	AG	17,7
Dip2	TT	AA	GG	13,3
Dip3	TC	GG	AG	10,8
Dip4	TT	GG	AA	9,5
Dip5	TC	GA	AG	9,5
Dip6	TC	GG	AA	7,6
Dip7	TT	GA	GG	5,1
Dip8	TC	GA	AA	5,1

Only frequencies >5% have been shown

Table 3. Demographic features of the 158 systemic lupus erythematosus patients studied and their genotype profile

	Entire Cohort (n=)	Mutation (n=) -1237 / +1174 / +2848	P value
Age (mean ±SD)	39.4 (±11.81)	38.22 / 41.97 / 39.49	0.759 / 0.185 / 0.951*
Ethnic Group			
- Caucasoid	29 (18,4%)	3 / 9 / 11	
- Afro-descendent	28 (19,9%)	1 / 5 / 8	0.624 / 0.201 / 0,486**
- Mulato	94 (61,7%)	5 / 14 / 14	
Gender (female)	154 (97.5%)	10 / 31 / 47	0.598 / 0.811 / 0.406**
Disease duration in years (median)	8.0	3 / 15 / 20***	0.364 / 0.610 / 0.890 *

** Kruskal Wallis test; ** Pearson's Chi-square test; *** Patients > median of disease duration.*

Table 4 – Clinical manifestations of the 158 systemic lupus erythematosus patients studied and their association with different polymorphism

Clinical features	number (%)	Mutated SNP (n) -1237 / +1174 / +2848	P-value*
Malar Rash	102 (64.6)	6 / 21 / 33	0.756 / 0.888 / 0.623
Discoid Rash	32 (20.3)	4 / 6 / 15	0.108 / 0.813 / 0.030
Photosensitivity	137 (86.7)	9 / 30 / 46	0.751 / 0.189 / 0.075
Oral ulcers	67 (42.4)	4 / 12 / 22	0.874 / 0.530 / 0.671
Arthritis	151 (95.6)	10 / 31 / 47	0.482 / 0.688 / 0.886
Serositis	39 (24.7)	2 / 5 / 10	0.723 / 0.183 / 0.403
- pericarditis	17 (10.7)	1 / 1 / 4	0.984 / 0.152 / 0.610
- pleuritis	34 (21.5)	2 / 4 / 8	0.935 / 0.216 / 0.374
Renal involvement[a]	50 (32.3)	2 / 10 / 16	0.391 / 1.000 / 0.848
CNS involvement	25 (15.8)	4 / 6 / 8	0.030 / 0.611 / 0.907
- Psychosis	19 (12)	4 / 3 / 6	0.005 / 0.606 / 0.955
- Seizures	13 (8.2)	1 / 4 / 3	0.833 / 0.325 / 0.518
Hematological involvement[b]	111 (71.6)	5 / 25 / 34	0.117 / 0.359 / 0.855

[a] *Renal involvement = Proteinuria >1g or creatinine above normal range secondary to SLE any time;* [b] *Hematological involvement = leucopenia <4.0 mm^3; lymphopenia <1.5 mm^3 and/or thrombocytopenia < 100.0 mm^3;* * *Pearson´s chi-square – significant p-value < 0.05.*

Table 5. Age (mean ± SD) of some clinical manifestation subgroups according to different genotypes of *TLR9* genes.

	Entire Cohort	Position -1237		Position +1174		Position +2848	
		Mutant	Non mutant	Mutant	Non mutant	Mutant	Non mutant
Psychosis n=19	37.4 (± 13.4)	31.5 (± 12.4)	38.9 (± 13.6)	26.7 (±5.9)	39.4 (± 13.6)	35.2 (±11.6)	38.4 (±14.5)
Discoid rash n=32	44.2 (± 10.8)	49.3 (±0.58)	43.7 (±11.3)	43.4 (± 13.4)	44.4 (±10.5)	43.9 (±11.3)	44.6 (±10.7)
Renal Involvement n=50	34.9 (± 11.6)	23.5 (±4.9)	35.4 (± 11.6)	37 (± 12.2)	34.4 (± 11.6)	31.6 (± 13.0)	36.4 (± 10.8)

Table 6. Disease Duration (Median – Quartile 25 - 75) of some clinical manifestation subgroups according to different genotypes of *TLR9* genes.

	Entire Cohort	**Position -1237**		**Position +1174**		**Position +2848**	
		Mutant	Non mutant	Mutant	Non mutant	Mutant	Non mutant
Psychosis n=19	9 (3 – 14)	4.5 (2.3 – 9.8)	9 (6 – 14)	8 (6 – 14)	9 (3 – 13.8)	10.5 (5.3 – 15)	9 (2.5 – 12.5)
Discoid rash n=32	8 (4 – 18)	10.5 (3 – 21)	8 (4 – 17.75)	5,5 (1 – 15.5)	8 (4 – 18.5)	4 (3 – 18)	9 (6.5 – 20)
Renal Involvement n=50	7 (4 – 11)	4 (2 – 6)	7 (4.3 – 11)	8 (4.5 – 11.8)	7 (4 – 11)	7 (5 – 12.5)	7 (4 – 11)

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5. Discussão

LES é uma doença autoimune multissistêmica cuja exata etiologia permanece incerta, contudo evidências demonstram a importância dos fatores genéticos para o desenvolvimento da doença (RAHMAN & ISENBERG, 2008; SESTAK *et al.* 2007). Tendo em vista a relevância da genética para o LES, muitos são os trabalhos que obtiveram sucesso encontrando associações entre determinado gene e a susceptibilidade a doença. Genes de HLA classe II, de proteínas do sistema complemento, de algumas citocinas, como TNF-alfa, TNF-beta e IL-10, já foram descritos como associados ao LES (YANG *et al.* 2004; HARTUNG *et al.* 1992; WILSON *et al.* 1994; D'Alfonso *et al.* 2002). Contudo acredita-se que é necessário haver a combinação de quatro a cinco genes para um paciente vir a desenvolver a patologia (SCHUR, 1995). Não existem, porém, muitos trabalhos na literatura correlacionando o risco de desenvolver determinada manifestação clínica em genes em pacientes com LES. Dois trabalhos descreveram que polimorfismos nas regiões que codificam os receptores das porções Fc de imunoglobulinas G estão correlacionados com o surgimento mais precoce de alguns sintomas como nefrite, artrite, anormalidades hematológicas, entre outros (MANGER *et al.*, 1998; YUN *et al.*, 2001). O trabalho de Liu *et al.* em 2002 descreve a associação de polimorfismos no receptor de estrogênio com a nefrite lúpica (LIU *et al.* 2002).

O TLR9 desempenha uma função primordial no sistema imune inato reconhecendo sequências CpG não metiladas de DNA de procariotos, mas estudos mostram que esta molécula também reconhece DNA endógeno de pacientes com LES, tornando essa

molécula uma das possíveis explicações para o surgimento dos sintomas no LES (LAFYATIS & MARSHAK-ROTHSTEIN, 2007). Baseado nessa hipótese, pesquisadores tentaram identificar se polimorfismos genéticos no TLR9 são associados com a susceptibilidade ao lúpus, obtendo resultados inconsistentes, sendo esses estudos realizados em populações com diferentes *backgrounds* genéticos (TAO *et al.* 2007; XU *et al.* 2009; DEMIRCI *et al.* 2007; De JAGER *et al.* 2006; NG *et al.* 2005; HUR *et al.* 2005). Tendo em vista que no presente estudo não foi incluído um grupo controle com pacientes sadios do Brasil, não foi possível avaliar se polimorfismos no gene do TLR9 está associada a susceptibilidade ao LES em um grupo étnico distinto.

O principal objetivo desse estudo foi de descrever pela primeira vez a prevalência dos polimorfismos de TLR9 em pacientes com LES no Brasil (tabela 2) e avaliar se essas modificações genéticas estão associadas com alguma manifestação clínica ou complicação da doença. Foi encontrado que o genótipo CC na posição -1237 está associado com o envolvimento neurológico ($p<0,05$), mais precisamente com a psicose ($p<0,01$), e que o genótipo GG na posição +2848 está correlacionado com eritema discóide ($p<0,05$). Dos seis estudos realizados previamente envolvendo polimorfismos genéticos do TLR9 em LES, Ng *et al.* (2005), Hur *et al.* (2005) e De Jager *et al.* (2006) foram os únicos a tentarem correlacionar esse gene com sintomas clínicos e não conseguiram encontrar nenhuma associação.

Todos os SNPs avaliados nesse estudo demonstraram estar em equilibrio de Hardy-Weinberg, mas os alelos nas posições -1237 e +1174, e nas posições +1174 e +2848 se apresentaram em uma freqüência maior que a esperada, demonstrando estar em

desequilíbrio de ligação. Tendo em vista que neste trabalho não foram estudadas as famílias dos pacientes, não foi possível obter a origem parental de cada alelo, tornando o conceito de desequilíbrio de ligação, não aplicável neste caso. Desta forma, acredita-se que a maior freqüência dos alelos citados acima foi encontrada devido ao acaso. Contudo, visto que os SNPs encontrados em associação com os sinais clínicos não produzem formas diferentes de TLR9, já que uma das mutações estudadas encontra-se na região promotora, posição -1237, e a outra apesar de acontecer na região codificadora, mais precisamente no segundo éxon, não leva a mudança no aminoácido (mutação silenciosa), posição +2848, a hipótese de desequilíbrio de ligação desses SNPs com um polimorfismo não avaliado nesse estudo, como razão desses achados, não pode ser descartada.

Outra provável explicação biológica para uma mutação na região promotora (posição -1237) levar ao surgimento de sintomas recai na função que essas regiões possuem para a transcrição do RNA mensageiro (RNAm). Na região promotora do gene acontece a ligação de proteínas diretamente envolvidas na transcrição do RNA mensageiro codificado pelo gene em questão. Desta forma, uma mutação pontual nesta região pode influenciar na produção final da proteína, no caso, a TLR9.

Apesar do SNP na posição +2848 não levar a modificação do aminoácido que o códon traduz, a mudança da sequência nucleotídica pode levar a diminuição da proteína através da redução da velocidade na síntese protéica. As concentrações intracelulares dos RNAs transportadores (RNAt) variam de acordo com os níveis de utilização dos mesmos. Ao modificar a sequência do códon, apesar da substituição não mudar o

aminoácido traduzido, a proteína, no caso TLR9, pode se encontrar em menor concentração na célula devido ao RNAt, que possui a sequência do novo anticódon, poder estar em menor quantidade que o RNAt original. Outras possíveis explicações para mudanças na expressão do TLR9 podem estar relacionadas a eventos relacionados aos mecanismos de *splicing*, através de snRNAs, ou envolvidos ao silenciamento do RNA mensageiro, através de RNAs de interferência.

Ao avaliar os grupos de pacientes com o polimorfismo genético associados aos sintomas, com significância estatística, em relação a idade e tempo de doença, foi observado que pacientes com psicose e com mutação na posição -1237 eram mais jovens e tinham um menor tempo de doença que os pacientes com psicose e sem o polimorfismo. Similarmente, pacientes com eritema discóide e com mutações na posição +2848 apresentavam um menor tempo de doença que os pacientes com o sinal clínico e sem o polimorfismo (tabelas 5 e 6). Desta forma, é possível chegar a conclusão que os SNPs reportados podem estar associados com um início mais precoce das manifestações clínicas avaliadas.

As limitações deste trabalho são que este não é um estudo de base populacional, desta forma, os resultados encontrados podem não representar o que acontece de fato na cidade de Salvador, e que a população deste estudo é composta por pacientes já atendidos por uma unidade reumatológica e não representa uma série de novos pacientes sem tratamento prévio. Desta forma, a correlação dos polimorfismos de TLR9 com os anticorpos anti-dsDNA, que seria de grande relevância já que a presença da

proteína foi correlacionada com o surgimento desses anticorpos, não pôde ser realizada, já que estes se relacionam diretamente com a atividade de doença.

Outra potencial limitação se refere ao desenho de estudo escolhido. A escolha ideal do grupo controle fica limitada quando ocorre a possibilidade dos pacientes virem a desenvolver determinado sintoma no futuro, no caso dos resultados encontrados neste estudo, psicose ou eritema discóide.

6. Conclusões

- Os polimorfismos -1237 C>T, +1174 A>G e +2848 G>A no gene do TLR9 são freqüentes em pacientes Brasileiros com LES;
- SNPs nas posições -1237 (C>T) e +2848 (G>A) estão associados com envolvimento do SNC e eritema discóide em pacientes com LES no Brasil;
- SNPs nas posições -1237 (C>T) e +2848 (G>A) estão associados com um início precoce do envolvimento do SNC e eritema discóide em pacientes com LES no Brasil;
- Mais estudos são necessários para confirmar as associações encontradas neste trabalho e uma pesquisa mais aprofundada é necessária para explorar os efeitos patogênicos do TLR9 nessas complicações em pacientes com LES.

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8. Anexos

Artigos publicados no período do mestrado

Pesquisa de Anticorpos Antinucleossoma em Lúpus Eritematoso Sistêmico

Detection of Antinucleosome Antibodies in Systemic Lupus Erythematosus

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RESUMO

Objetivos: determinar a freqüência dos anticorpos antinucleossoma (AN) em lúpus eritematoso sistêmico (LES) e avaliar a associação desses anticorpos com a atividade de doença. **Métodos:** estudo de corte transversal em que foram estudados pacientes com diagnóstico de LES baseado nos critérios do Colégio Americano de Reumatologia. Utilizou-se o SLEDAI como instrumento de avaliação de atividade de doença. A pesquisa de anticorpos AN foi realizada pela técnica de ELISA (INOVA Diagnostics Inc). Pacientes com diagnóstico de miosite e esclerose sistêmica (ES) foram também estudados para avaliação da *performance* do teste. **Resultados:** foram estudados 82 pacientes com LES, sendo 81 do sexo feminino, com idade média de 35 ± 11,7 anos. Anticorpos AN foram detectados em 48 pacientes com LES (58,5%), em três pacientes com miosite (21,4%) e em dois pacientes com ES (14,2%), o que determinou sensibilidade e especificidade de 58,5% e 82,14%, respectivamente. Considerando-se um ponto de corte em 40 U, os anticorpos AN foram detectados em 44 pacientes com LES (53,65%), em dois pacientes com miosite (13,33%) e em um paciente com ES (6,66%), o que determinou sensibilidade e especificidade de 53,65% e 90%, respectivamente. Não se observou correlação dos títulos dos anticorpos AN e a atividade de doença (SLEDAI), embora tenha sido observada correlação entre anti-DNA e escore de SLEDAI nessa mesma população ($r = 0,42$; $p < 0,005$). **Conclusões:** No presente estudo, observaram-se moderada sensibilidade e alta especificidade dos anticorpos AN para o diagnóstico de LES. No entanto, não se verificou associação desses anticorpos com os parâmetros de atividade da doença, sugerindo que a pesquisa de tais anticorpos seria de valor limitado na prática reumatológica.

Palavras-chave: lúpus eritematoso sistêmico, antinucleossoma, SLEDAI.

ABSTRACT

Objective: *to determine the frequency of antinucleosome (AN) antibodies in systemic lupus erythematosus (SLE) and their association with disease activity.* ***Methods:*** *cross-sectional study to evaluate patients with diagnosis of SLE based on the American College of Rheumatology criteria. SLEDAI score was used as a disease activity index. AN antibodies were tested by ELISA (INOVA Diagnostics Inc). Systemic sclerosis (SSc) and myositis patients were also studied to determine the diagnostic performance of the ELISA system.* ***Results:*** *a total of 82 SLE patients, 81 female, mean age 35 ± 11.7 years were included in the study. AN antibodies were positive in 48 SLE samples (58.5%), three with myositis (21.4%) and two with SSc (14.2%), determining a sensitivity and specificity of AN antibodies for the diagnosis of SLE of 58.5% and 82.14%, respectively. Utilizing a cut off of 40 U, test was positive in 45 SLE samples (53.65%), two with myositis (13.33%) and one with SSc (6.66%), determining a sensitivity and specificity of AN antibodies for the diagnosis of SLE of 53.65% and 90%, respectively. There were no correlation between AN antibodies and SLEDAI scores. On the other hand, it was observed a positive correlation between anti-DNA antibodies and disease activity ($r = 0.42$; $p < 0.005$).* ***Conclusions:*** *in the present study it was demonstrated a high specificity and moderate sensitivity of AN antibodies for the diagnosis of SLE. However, the lack of association with disease activity suggests that it has limited value in rheumatologic practice.*

Keywords: *systemic lupus erythematosus, antinucleosome, SLEDAI.*

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INTRODUÇÃO

O lúpus eritematoso sistêmico (LES) é uma doença auto-imune, de etiologia não completamente esclarecida, que concorre com significativa morbi-mortalidade para os pacientes acometidos, em sua maioria mulheres jovens. Sua classificação, proposta pelo Colégio Americano de Reumatologia (ACR), baseia-se na presença de quatro dos 11 critérios em qualquer fase de evolução da doença[1]. No entanto, freqüentemente há dificuldades em se estabelecer o diagnóstico em vista de sua apresentação clínica polimórfica, bem como a inespecificidade dos exames diagnósticos.

Vários estudos em andamento têm buscado exames mais sensíveis e específicos para o diagnóstico do LES, e estudos de fisiopatogenia têm apontado o nucleossoma como um auto-antígeno que, a partir de sua exposição pela apoptose, torna-se capaz de induzir a produção de anticorpos, mediadores das lesões teciduais no LES[2-5]. À luz desse conhecimento, tem-se buscado o significado da pesquisa de anticorpos antinucleossoma (AN) como teste diagnóstico para o LES. O objetivo do presente estudo foi determinar a relevância dos anticorpos antinucleossoma (AN) no LES e avaliar a associação desses anticorpos com a atividade da doença.

MATERIAL E MÉTODOS

Foram estudados pacientes com diagnóstico de LES baseado nos critérios da ACR[1] em acompanhamento no Serviço de Reumatologia do Hospital Santa Izabel, Salvador, Bahia. Também foram estudados pacientes com diagnóstico de esclerose sistêmica (ES)[6] e miosite[7,8] segundo os critérios previamente publicados, acompanhados no mesmo serviço. Todos os pacientes assinaram o Termo de Consentimento Livre e Esclarecido antes de entrarem no estudo.

Os pacientes portadores de LES foram avaliados quanto à atividade da doença utilizando-se como instrumento o *Systemic Lupus Erythematosus Disease Activity Index* (SLEDAI)[9]. A pesquisa de anticorpos anti-DNA nativo foi realizada por imunofluorescência indireta, utilizando-se a *Crithidia luciliae* como substrato[10]. A pesquisa de anticorpos antinucleossoma utilizou o método de ELISA por meio de um *kit* adquirido comercialmente (INOVA Diagnostics Inc., San Diego, CA). Tal método tem como substrato a cromatina purificada de timo bovino, e foi utilizado como ponto de corte o valor de 20U para expressar os resultados como positivos, como sugerido pelo fabricante. A análise estatística foi realizada utilizando-se o programa *Statistical Package for the Social Sciences* (SPSS Chicago – IL, versão 9.0, 1998). As variáveis nominais foram descritas sob a forma de freqüências, e as variáveis intervalares, sob a forma de média ± desvio-padrão. Utilizou-se o teste *t de Student* para comparar médias; a associação entre variáveis qualitativas foi estudada pelo teste de qui-quadrado ou exato de Fisher quando indicado, e a correlação entre variáveis contínuas foi estudada pelo teste de Pearson. Para todos os testes estatísticos, considerou-se significância o valor de $p < 0,05$. Calcularam-se sensibilidade, especificidade e valor preditivo positivo dos AN para o diagnóstico de LES utilizando-se um programa específico: (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTADOS

Foram estudados 112 pacientes, sendo 82 com diagnóstico de LES e 30 controles, que envolviam 15 pacientes com diagnóstico de miosite e 15 com ES. O grupo de LES era composto, em sua maioria, de pacientes do gênero feminino (81); tinha idade média de 35,0 ± 11,7 anos, tendo variado de 13 a 62 anos; e o tempo de doença variou de 2 a 384 meses, com média em 78,2 ± 76,1 meses. As características clínicas e laboratoriais, segundo os aspectos avaliados pelo SLEDAI, estão apresentadas nas Tabelas 1 e 2. A avaliação da atividade de doença revelou um escore SLEDAI médio de 11,6 ± 5,8 e escores mínimo e máximo de 2 e 30, respectivamente. Considerando-se arbitrariamente os pacientes em atividade aqueles que apresentavam SLEDAI maior do que 4, observou-se que 78 pacientes (95,12%) apresentavam doença "ativa". O escore médio dos pacientes em atividade foi de 12,12, tendo variado de 5 a 30; e dos pacientes inativos foi de 2,75, tendo variado de 2 a 4.

Tabela 1
Freqüência de sinais e sintomas presentes na avaliação dos 82 pacientes com lúpus eritematoso sistêmico – critérios avaliados de acordo com a definição do SLEDAI

Sinais e sintomas	N (%)
Febre	24 (29,3%)
Alopecia	70 (85,4%)
Rash malar	46 (56,1%)
Lesões de mucosa	25 (30,5%)
Artrite	44 (53,7%)
Pleurite	3 (3,7%)
Pericardite	1 (1,2%)
Glomerulonefrite	23 (28,0%)
Vasculite	7 (8,5%)
Psicose	1 (1,2%)

Tabela 2
Freqüência das alterações laboratoriais presentes na avaliação dos 82 pacientes com lúpus eritematoso sistêmico – critérios avaliados de acordo com a definição do SLEDAI

Alterações laboratoriais	N(%)
Leucopenia	44 (53,7%)
Plaquetopenia	12 (14,6%)
Proteinúria	22 (26,8%)
Hematúria	15 (18,3%)
Cilindrúria	14 (17,1%)
Leucocitúria	6 (7,3%)
Anticorpos anti-DNA	31 (37,8%)
Diminuição de C3	18 (22,0%)
Diminuição de C4	30 (36,5%)

Para todos os pacientes envolvidos no estudo, foi feita a pesquisa de anticorpos AN, tendo sido encontrada positividade em 48 (58,5%) em LES, dois (14,2%) dos pacientes com ES e três (21,4%) com miosite, correspondendo a uma sensibilidade de 58,5%, especificidade de 82,14% e valor preditivo positivo do teste para diagnóstio de LES de 90,56% nessa população estudada. Uma vez que o *cut off* desse sistema de ELISA não foi ainda determinado na população brasileira, optamos por analisar os resultados utilizando arbitrariamente um ponto de corte mais alto. Assim, considerando-se o ponto de corte em 40 U, encontramos positividade em 44 (53,65%) em LES, um (6,66%) dos pacientes com ES e dois (13,33%) com miosite, correspondendo a uma sensibilidade de 53,65%, especificidade de 90% e valor preditivo positivo do teste para diagnóstico de LES de 93,61% nessa população estudada. Adicionalmente, a média dos títulos dos anticorpos AN foi maior nos pacientes com LES: 86,3 U, comparando-se à dos pacientes com ES ou miosite: 45,1 U ($p < 0,05$). Ainda do ponto de vista de valor diagnóstico, observou-se menor freqüência de positividade de anticorpos anti-DNA do que de anticorpos AN no grupo de LES: 31 (37,8%).

Buscando a associação entre anticorpos AN e os parâmetros clínicos e laboratoriais avaliados pelo SLEDAI, não encontramos significância com nenhum sintoma ou alteração laboratorial presente, utilizando-se os dois diferentes pontos de corte, exceto para a associação entre febre e presença de AN ($p = 0,01$), com o ponto de corte de 20 U (Tabela 3). O título médio dos anticorpos AN foi de 55,83 ± 45,87 U nos pacientes ativos e de 25,05 ± 22,85 U nos inativos. Não houve diferença estatisticamente significativa entre a média dos títulos de AN dos pacientes ativos e inativos ($p = 0,65$), embora tal análise deva ser interpretada com cuidado, uma vez que a maioria dos pacientes estava no grupo "ativo". Adicionalmente, não encontramos correlação entre anticorpos AN e anti-DNA nem escore de SLEDAI, embora houvesse uma correlação direta, moderada e estatisticamente significativa entre anti-DNA e escore de SLEDAI nessa mesma população ($r = 0,42$; $p = 0,005$) (Tabela 4) e ($r = 0,31$; $p = 0,003$) para um ponto de corte de 40 U.

Tabela 3
Análise da associação da presença de anticorpos antinucleossoma e diferentes manifestações da doença nos 82 pacientes com lúpus eritematoso sistêmico. Ponto de corte para expressar positividade de AN de 20 U

Manifestação	Valor do p (qui-quadrado)
Febre	**0,01**
Alopecia	0,61
Aftas	0,08
Artrite	0,28
Pleurite	0,62
Pericardite	0,41
Vasculite*	0,31
Psicose	0,41
Glomerulonefrite**	0,53

*Vasculite: lesões cutâneas compatíveis com vasculite.
**Glomerulonefrite: presença de proteinúria > 0,5 g/24 horas associada ou não a hematúria, hipertensão arterial e perda de função renal.

Tabela 4
Análise da correlação entre os títulos dos anticorpos antinucleossoma (AN), anticorpos anti-DNA e escore de SLEDAI nos 78 pacientes com lúpus eritematoso sistêmico em atividade (Pearson). Ponto de corte para expressar positividade de AN de 20 U

	Anti-DNA	SLEDAI
AN	r = 0,16	r = 0,17
	p = 0,13	p = 0,13
Anti-DNA		r = 0,42
		p < 0,001

DISCUSSÃO

O nucleossoma é a unidade fundamental da cromatina. Durante o processo da apoptose, a cromatina sofre uma clivagem por ação de endonucleases, expondo os nucleossomas, que se comportam como antígenos, sendo capazes de induzir a formação de anticorpos[4,11,12]. Sabendo-se que a apoptose

está aumentada no contexto do LES, espera-se que se identifiquem anticorpos AN nesses pacientes[13]. À semelhança do presente estudo, outros trabalhos têm demonstrado a presença de anticorpos AN não só em LES, mas também em outras colagenoses, porém com maior freqüência e com títulos mais altos naqueles com LES[13]. Também à semelhança de nossos resultados, a freqüência de anticorpos AN tem sido maior do que a de anticorpos anti-DNA nas populações de LES estudadas, demonstrando-se a positividade de anticorpos AN em muitos casos em que o anti-DNA é negativo[11,15-18]. Por outro lado, Julkunen *et al.*[14] não encontraram maior sensibilidade dos anticorpos AN, comparando-se com os exames tradicionalmente realizados para o diagnóstico de LES.

No presente estudo, optamos por utilizar 20 U como ponto de corte para expressar a positividade dos AN, conforme as recomendações do fornecedor. Porém, tivemos o cuidado de realizar a análise dos dados utilizando também um *cut off* mais alto, de 40 U, uma vez que não é conhecido o comportamento desses anticorpos na população normal brasileira. Curiosamente, os resultados não diferiram significativamente daqueles utilizando um ponto de corte de 20 U.

Em vista da dificuldade freqüente no diagnóstico e na identificação de atividade no seguimento desses pacientes, diversos estudos têm buscado novos exames diagnósticos que permitam tal avaliação. Assim, em estudo previamente realizado no nosso serviço, avaliou-se o papel da dosagem sérica da enzima adenosina deaminase como marcador de atividade de doença e concluiu-se que não houve correlação da dosagem dessa enzima e o escore de SLEDAI[19]. Alguns trabalhos também têm demonstrado correlação dos anticorpos AN com parâmetros conhecidos de atividade de doença. Desse modo, dois trabalhos estudando anticorpos AN em LES na população brasileira encontraram sua associação com atividade de doença, particularmente renal[20,21]. Por outro lado, Quattrocchi *et al.*[22], estudando anticorpos AN em 47 casos de LES e em 62 pacientes com outras colagenoses, tais como artrite reumatóide, doença mista do tecido conjuntivo, ES e síndrome de Sjögren, além de 22 controles sadios, observaram que, apesar de confirmarem a alta sensibilidade desse teste para LES, não encontraram correlação com atividade de doença em LES, avaliada pelo ECLAM. Tais diferenças podem ser atribuídas a razões metodológicas, particularmente quanto ao instrumento utilizado na avaliação de atividade ou ao método de detecção dos anticopos AN.

Em conclusão, na nossa experiência, embora a pesquisa de anticorpos AN seja um teste de moderada sensibilidade e alta especificidade para o diagnóstico de LES, a não-associação com atividade de doença parece não justificar a pesquisa rotineira destes na prática reumatológica.

Declaramos a inexistência de conflitos de interesse.

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Anti-C1q Antibodies: Association With Nephritis and Disease Activity in Systemic Lupus Erythematosus

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Background: Anti-C1q antibodies have been described in systemic lupus erythematosus (SLE) as well as in other connective tissue diseases. They have been considered as a marker for disease activity and presence of nephritis.

Objective: The aim of this study was to determine the prevalence of anti-C1q antibodies in Brazilian lupus patients as well as analyze their association with different clinical and serologic parameters.

Methods: Sera from 81 SLE patients, based on the American College of Rheumatology (ACR) criteria, were collected from a lupus referral outpatient clinic in Salvador, Brazil. Antibodies to C1q were detected by an enzyme-linked immunoassay (ELISA) kit and antibodies to other cellular antigens identified by indirect immunofluorescence on HEp-2 cell substrate (ANA), or *Crithidia luciliae* (dsDNA), and to nucleosome by ELISA. A cutoff of 20 U was established for anti-C1q and antinucleosome assays.

Results: Anti-C1q antibodies were detected in 39.5% (32/81) of SLE sera. The presence of anti-C1q antibodies was associated with proteinuria ($P = 0.028$) but not with other laboratory or clinical features, such as antinucleosome or anti-dsDNA antibodies, hematuria, urinary casts or renal failure, leukopenia, pericarditis, pleuritis, malar rash, seizures, and psychosis. There was a positive correlation between the titers of anti-C1q antibodies and the systemic lupuis erythematosus disease activity index (SLEDAI) score ($r = 0.370$; $P = 0.001$).

Conclusion: This study in Brazilian SLE patients confirms previous findings of the association of anti-C1q antibodies with nephritis and disease activity. J. Clin. Lab. Anal. 23:19–23, 2009.

Key words: autoantibodies; anti-C1q antibodies; systemic lupus erythematosus; nephritis

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystemic disorder involving several autoantibodies with a potential etiopathogenic role, such as anti-dsDNA, anti-phospholipid, and anti-SSA (Ro). Likewise, impairment of clearance of immune complexes and apoptotic cells has been considered as an important factor contributing to the development of the disease. Moreover, deficiency of complement components has also been classically associated with the development of SLE. One case of this type is homozygous C1q deficiency (1).

More recently, several studies have demonstrated the presence of antibodies directed to this component of

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complement in SLE (2–5), as well as in other connective tissue diseases (6,7) and hepatitis C infection (7). Nevertheless, in SLE their presence has been associated with disease activity, particularly renal flares of lupus nephritis (5,8,9). The role of C1q in clearance of apoptotic cells and immune complexes is the probable mechanism linking its deficiency or inhibition with autoimmune diseases (10,11).

The aim of this study was to investigate the frequency of anti-C1q antibodies in a group of Brazilian SLE patients and evaluate their association with different clinical features of the disease.

MATERIALS AND METHODS

Patient Enrollment

Unselected SLE patients were collected from a lupus referral outpatient clinic in Salvador, Brazil. The diagnosis of SLE was based on the ACR criteria (12). Disease activity was evaluated by SLEDAI (13). Patients infected with Virus C hepatitis were not included in the study.

Proteinuria was defined as 500 mg/24 hr or higher in the last 10 days, renal dysfunction as any increase in creatinine value at any time in the history, and renal involvement as any of the two above variables. Other clinical features were labeled if present at any time in the course of the disease.

The study had the approval of the Ethics Review Board of our institution and each patient signed a consent form on enrollment.

Laboratory Procedures

Aliquots of sera were stored at −20°C until needed and all autoantibody testing was performed at the Laboratory of Molecular Biology, Gonçalo Muniz Foundation, Bahia, Brazil. This included an autoantibody analysis by indirect immunofluorescence on HEp-2 substrate (ANA) and *Crithidia luciliae* (anti-dsDNA). Antibodies to nucleosome were measured by enzyme-linked immunoassay (ELISA) (INOVA Diagnostics Inc., San Diego, CA).

Anti-C1q antibodies by ELISA: An ELISA kit was used to detect anti-C1q as follows. Patient samples were diluted 1/100 and then added to each well. After a 30-min incubation, the wells were washed with high-ionic-strength buffer to remove immunoglobulins bound to the globular head region of C1q, leaving behind specific anti-C1q antibodies. Then horseradish peroxidase coupled to anti-human IgG conjugate supplied with the kit was used as the secondary antibody. After developing the reaction with the chromogen supplied, the absorbance was read at 450 nm. Values <20 U were considered as negative, and positives were classified as "weak" (20–39 U), "moderate" (40–80 U), and "strong" (>80 U) as suggested by the manufacturer.

Statistical Analysis

All clinical and laboratory data were entered into and extracted from SPSS for Windows (version 14.0). Some results were expressed as mean ± standard deviation. The Kolmogorov–Smirnov was utilized to test the normality of the variables. The Mann–Whitney test was used to compare means. Correlation coefficient was investigated by Spearman test. The association between qualitative variables was evaluated by Yates' χ^2 with correction or Fisher's exact test, when indicated, considering $P<0.05$ as statistically significant.

RESULTS

SLE Demographics and Main Clinical Features

The unselected SLE population studied included 80 (98.7%) women and only 1 (1.3%) man with a mean age of 34 (±11) years. The median disease duration was 49 months (range 2–384). The distribution of race was 40.7% Mulatto, 29.6% Caucasoid, and 29.6% Black (Table 1). The main clinical features of the studied population are presented in Table 2.

Clinical and Serological Correlate of Anti-C1q Antibodies

When the ELISA method was used to detect anti-C1q antibodies in the SLE sera, a prevalence of 39.5% (32/81) was observed. The comparative clinical and laboratory data for anti-C1q-positive and anti-C1q-negative patients are presented in Table 3. This correlation refers to the presence of the manifestation at the time of visit or in the preceding 10 days as defined by the SLEDAI criteria.

The presence of anti-C1q antibodies was associated with proteinuria in the last 10 days, defined as higher than 500 mg/24 hr ($P=0.028$). This association was stronger when one considers proteinuria at any time in the history ($P=0.01$). In addition, there was a positive correlation between the titers of anti-C1q antibodies and the SLEDAI score ($r=0.370$; $P=0.001$) and the mean

TABLE 1. Demographic Features of 81 Systemic Lupus Erythematosus Patients

Demographic features	Results
Age (mean ± SD)	34 (±11) yr
Race (*Mulatto*/Caucasoid/Black)	40.7/29.6/29.6%
Gender (female)	80 (98.7%)
Disease duration in months (median) (range)	49 months (range 2–384)

SLEDAI score was higher in the anti-C1q-positive group ($P = 0.008$). On the other hand, there was no association with any of the clinical parameters such as arthritis, malar rash, mucosal ulcers, cutaneous vasculitis, pleuritis, urinary casts, hematuria, renal failure, fever, leukopenia, thrombocytopenia, presence of antinucleosome, anti-dsDNA antibodies, or lower levels of C3 or C4 of the complement cascade. Notably, there was no correlation between the titers of antinucleosome and anti-C1q antibodies ($r = 0.170$; $P = 0.129$).

TABLE 2. Main Clinical Features of the Studied Population of Systemic Lupus Erythematosus

Features	%
Arthritis	100
Fever	95.1
Photosensitivity	90.1
Malar rash	82.7
Mucosal ulcers	72.8
Discoid rash	16
Raynaud's phenomenon	67.9
Cutaneous vasculitis	22.2
Pericarditis	8.6
Pleuritis	14.8
Proteinuria[a]	56.8
Urinary casts	29.6
Renal dysfunction[b]	17.3
Hypertension	27.2
Psychosis	7.4
Seizures	7.4
Leukopenia[c]	64.2
Thrombocytopenia[d]	17.3
Hemolytic anemia	17.3

The percentage refers to the presence at any time in history.
[a]Proteinuria (>500 mg/24 hr).
[b]Renal dysfunction: any increase in creatinine value at any time in the history.
[c]Leukopenia: <4,000 cells/mm^3.
[d]Thrombocytopenia: <100,000 cells/mm^3.

DISCUSSION

In this study, a prevalence of 39.5% of anti-C1q antibodies was found in Brazilian SLE patients. If one takes into consideration only those with proteinuria higher than 500 mg/24 hr in the last 10 days the frequency of anti-C1q increases to 59%. This figure is similar to that observed by Sinico et al., who found anti-C1q antibodies in 27 of 61 (44%) SLE patients, and in 60% of patients with lupus nephritis, when compared with only 14% of SLE patients without nephropathy ($P<0.05$) (5).

Curiously enough, although an association of anti-C1q and proteinuria identified in the last 10 days was found, as well as a positive correlation with SLEDAI score, no association of these antibodies was found with renal dysfunction, classified as any increase in creatinine value in the present or in the past. It may suggest that the detection of anti-C1q antibodies is a marker for disease activity, particularly in a renal site. On the other hand, the authors were unable to demonstrate any association of anti-C1q with anti-dsDNA or antinucleosome antibodies. Although similar results were observed in some studies (8,14), others found discordant results. Thus, Mosca et al. stated that anti-C1q antibodies do not seem to be related to the occurrence of flares during pregnancy (15). Braun et al. (6) found anti-C1q antibodies to be significantly correlated with anti-dsDNA. Although Oelzner et al. (14) found a positive

TABLE 3. Clinical and Serological Correlation of Anti-C1q Antibodies in 81 Systemic Lupus Erythematosus Patients[a]

Features	Entire group ($n = 81$) (%)	Anti-C1q positive ($n = 32$) (%)	Anti-C1q negative ($n = 49$) (%)	P value
Arthritis	54.3	62.5	49	0.232
Malar rash	56.8	56.3	57.1	0.937
Mucosal ulcers	30.9	31.3	30.6	0.952
Cutaneous vasculitis	8.6	6.3	10.2	0.698
Pleuritis	3.7	3.1	4.1	1.000
Proteinuria (>500 mg/24 hr)	27.2	40.6	18.4	0.028
Urinary casts	16	18.8	14.3	0.593
Hematuria	17.3	25	12.2	0.138
Fever	29.6	28.1	30.6	0.811
Leukopenia (<4,000 cells/mm^3)	54.3	50	57.1	0.528
Thrombocytopenia (<100,000 cells/mm^3)	14.8	15.6	14.3	0.868
Antinucleosome antibodies	53.1	53.1	53.1	0.996
Anti-dsDNA antibodies	38.3	40.6	36.7	0.725
C3 below normal range	22.2	31.3	16.3	0.114
C4 below normal range	37	43.8	32.7	0.312

[a]Correlation refers to the presence of the manifestation at the time of visit or in the preceding 10 d, as defined by the SLEDAI criteria. SLE, systemic lupus erythematosus.

correlation with the SLEDAI score, they did not find association with lupus nephritis.

It is still intriguing how antibodies to C1q could cause renal disease in SLE. An indirect evidence for the role of these antibodies in the pathogenesis of lupus nephritis is the observation by Chen et al. who found higher titers of anti-C1q in patients with lupus nephritis and C1q deposition in the kidney tissue (16). Other reasonable explanations would be that the autoantibodies inhibit the removal of apoptotic cells secondary to complement deficiency induced by these antibodies; they block the clearance of C1q-containing immune complexes, allowing them to deposit in the glomeruli, or they activate the complement cascade and consequently the inflammatory process (17–20).

On the other hand, the presence of anti-C1q antibodies has also been identified in the serum of patients with other connective tissue diseases without renal involvement or even in the normal population (21). Thus, the possibility that these antibodies may represent only an epiphenomenon cannot be entirely excluded. However, studies in animal models have suggested that anti-C1q may amplify complement activation, that is, when a monoclonal anti-C1q antibody alone was administered to mice, it was unable to cause renal damage, but if the level of C1q was increased in glomerulus by the previous interaction of other antibodies with glomerular antigens, the mice presented renal damage (19,20). These observations may suggest that in SLE, different from other conditions, anti-C1q antibodies have the potential to cause renal damage. The observation in this study, of no association between antinucleosome and anti-C1q antibodies, has been corroborated by others (22). In a previous study no association was found between antinucleosome and disease activity in SLE (23). Thus, these two antibodies seem to identify different subsets of SLE patients.

In conclusion, this study in Brazilian SLE patients confirms previous findings of the association of anti-C1q antibodies with nephritis and disease activity. Hence, the detection of such antibodies in SLE seems to be a useful tool for the management of these patients.

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SHORT COMMUNICATION

NK and NKT cell dynamics after rituximab therapy for systemic lupus erythematosus and rheumatoid arthritis

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Abstract Biomarkers of clinical response to rituximab (RTX) therapy and early predictors of outcome are still under investigation. We report a flow cytometric immunophenotyping analysis from peripheral blood leukocyte subpopulations of two patients with systemic lupus erythematosus (SLE) associated thrombocytopenia and one patient with rheumatoid arthritis (RA), before and after 6 weeks of treatment with RTX. Our results show a reduced population of $CD19^+$ expressing cells (B cells) after RTX treatment in all three patients. Increased frequency of peripheral regulatory $CD4^+CD25^{high}$ T cell subset and the $CD3^-CD16^-CD56^{bright}$ NK cell subset after RTX therapy were also observed in all patients, the latter being more pronounced in the SLE patient with sustained clinical response. In addition, an increased population of NKT cell subsets was observed in the patients with clinical response. This is the first evaluation of NK and NKT cells as biomarkers of clinical response after rituximab therapy in rheumatic diseases.

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Keywords Systemic lupus erythematosus · Rheumatoid arthritis · Natural killer cells · Rituximab · Leukocytes

Introduction

Rituximab (RTX) is a monoclonal antibody against CD20 that emerges as an important option of biologic therapy for autoimmune diseases. It was first approved for the treatment of non-Hodgkin lymphomas in 1997 by the Food and Drug Administration, United States. Its application in rheumatology was first evaluated for rheumatoid arthritis (RA) and later for other conditions including systemic lupus erythematosus (SLE), with promising results [1]. CD20 is a non-glycosylated surface membrane phosphoprotein restrictedly expressed in B lymphocytes before plasma cell differentiation. Importantly, since plasma cells are not a target for anti-CD20 antibodies, some autoantibodies may persist in serum even after depletion of B cells and clinical improvement of rheumatic diseases [1, 2].

The exact mechanism of how RTX influences the immune responses in autoimmune disorders leading to clinical remission is not known yet. B cell depletion and inhibition of antibody production are unlikely to explain all its therapeutic effects. RA has a poorly understood pathogenesis but the widely accepted view identifies rheumatoid factor as an epiphenomenon rather than an autoantibody directly implicated in disease [1]. Furthermore, there is not a clear association between clinical improvement after RTX therapy and B cell depletion, for either RA or SLE patients, or lowering titers of anti-double stranded DNA antibodies (dsDNA), for SLE patients [1–5] and, thus, research in RTX therapy in autoimmune diseases includes seeking new biomarkers that may predict or measure therapeutic response [6].

B lymphocytes are not viewed anymore as merely antibody producing cells. They may act as antigen presenting cells (APCs) being the major source of T lymphocyte activation in some models. Moreover, B lymphocytes may produce high amounts of cytokines with immunomodulatory effects [1, 2, 7]. Decreased T helper cell activation after RTX treatment has been suggested on the basis of lower frequency of costimulatory T cell molecule CD40L positive cells that has been associated with partial and complete remission in patients with lupus nephritis [3, 8]. Interestingly, flow cytometry analysis of peripheral blood leukocytes after RTX therapy have shown additional effects on other cell types other than B lymphocytes [2, 3, 7]. After RTX therapy, macrophages produce lower amounts of TNFα and exhibit increased expression of CD68, thus developing an APC phenotype as compensatory mechanism for the depletion of APC functioning B cells in RA [9]. Natural killer (NK) and NKT cells are recognized by their cytotoxic and regulatory effects, implicated in prevention of autoimmunity [10, 11]. For example, NKT cell frequency in peripheral blood is lower in patients with SLE when compared to healthy subjects [12] and decreased values of NKT cell counts are associated with higher levels of anti-dsDNA [13]. T $CD4^+CD25^{high}$ cells are important natural modulators of self-antigen T cell mediated responses [14]. While such natural regulatory T cells are reduced in patients with SLE [15, 16], inducible $CD4^+IL10^+$ regulatory T cell count increases in SLE patients probably as result of compensatory mechanism [15].

The aim of this study was to explore the relationship of the peripheral blood regulatory NK, NKT cell subsets and $CD4^+CD25^{high}$ T cells with clinical response after RTX therapy.

Patients

Case 1 (SLE)

A 21-year-old female patient had 4-year past history of SLE presenting during evolution: malar rash, photosensitivity, arthritis, lymphopenia, nephrotic syndrome and positive antinuclear antibodies. The patient was treated with oral corticosteroids and cyclophosphamide pulses for 2 years with clinical improvement and azathioprine was introduced as maintenance therapy. Later, she presented persistent thrombocytopenia and autoimmune hemolytic anemia refractory to higher dose of azathioprine (150 mg/day) and 20 mg/day of prednisolone. Therapy with RTX 375 mg/m^2 IV weekly for 4 weeks leads to an increase in platelet count reaching normal figure after 1 month. The patient has been followed up with no symptoms and with normal platelet count allowing discontinuation of azathioprine and corticosteroid. She has been out of medication for 17 months.

Case 2 (SLE)

A 42-year-old female patient had 3-year past history of SLE characterized by articular symptoms, photosensitivity, thrombocytopenia, leukopenia and presence of antinuclear antibodies. She had been unsuccessfully treated for 3 years with corticosteroids (oral and pulses), azathioprine, intravenous immunoglobulin and danazol for refractory thrombocytopenia. Treatment with RTX was introduced, following the same protocol as described above, when platelet count reached 25,000/mm^3. Thrombocytopenia responded well to RTX therapy; however, it recurred 9 months after initial therapy and a new course therapy is ongoing.

Case 3 (RA)

A 48-year-old female patient had 5-year past history of RA and secondary Sjögren's syndrome. Clinical features during evolution were symmetric poliarthritis of large and small joints, anemia, xerophtalmia, intermittent parotid gland enlargement, and positive rheumathoid factor and anticyclic citrullinated peptides (CCP) antibodies. She had been treated with methotrexate, leflunomide, cyclosporine, tacrolimus and sulphasalazine with no clinical improvement. After treatment with RTX, two doses of 1 g IV 2 weeks apart, with prednisone 1 mg/kg per day between doses, there was no clinical control of the disease albeit the patient exhibited rising levels of hemoglobin and lowering levels of erythrocyte sedimentation rate.

Methods

Blood samples

A 1-ml sample of peripheral blood was collected from each patient and normal healthy subject as control using ethylenediaminetetraacetic acid (EDTA). The patients were studied before and after rituximab therapy. Post-treatment evaluation was made after 6 weeks. After the collection, the whole peripheral blood (PB) was analyzed by flow cytometry. The study was approved by the Ethics Committee of our institution and all patients gave informed consent prior to the collection of blood.

Flow cytometry analysis of peripheral blood

Specific monoclonal antibodies (mAbs) were used with the following conjugated mouse anti-human mAbs in two- and three-color immunocytometric assays: FITC-conjugated

mAbs anti-CD3 (HIT3a), PE-conjugated mAbs included anti-CD19 (HIB19), anti-CD25 (IL-2Rα) (M-A251) and anti-CD56 (B159), Cy-conjugated mAbs CD4 (RPA-T4) and PE-Cy5 anti-CD16 (3G8). Isotype-matched negative-control mouse IgG_1-FITC, PE or Cy (MOPC-21) mAbs. All mAbs were purchased from Becton-Dickinson (Mountain View, CA, USA).

White blood cell phenotyping was performed according to the manufacturers instructions with the following modifications. In 12 × 75-mm polystyrene tubes 50 µl samples of peripheral blood, were added with 50 µl of mix containing FACS buffer (HBSS, 10% FCS, 0.01% sodium azide (Sigma-Aldrich), pH 7.2, 5 µl of each mAb) and incubated for 30–45 min at 4°C in the dark. Following the incubation, erythrocytes were lysed using 2 ml FACS lysing solution (BD Biosciences Pharmigen, San Diego, CA, USA.). The cells were washed twice in FACS buffer and resuspended in 200 µl FACS buffer. The cells were immediately acquired with.30,000 events on the FACSort flow cytometer (BD Biosciences) and the data analyzed using CellQuest Software (BD Biosciences). The results are expressed as a percentage of positive cells within the selected gate. Distinct gate strategies were used to analyze major and minor lymphocytes subsets described as follows: lymphocytes were gated according to their FSC versus SSC dot plot characteristics. Gated lymphocytes were further analyzed for their immunophenotypic features on dual fluorescence dot plot distributions. Analysis of NK cell subsets was performed within $CD3^-CD16^{\mp}CD56^+$ as previously described by Cooper et al. [17] and Vitelli-Avelar et al. [18]. Analysis of $CD56^{dim}$ (cytotoxic) and $CD56^{bright}$ (regulatory) NK cells was performed within $CD3^-CD16^+CD56^+$ NK cell subsets. Analysis of $CD56^{dim}$ and $CD56^{bright}$ NK cells was performed within $CD3^-CD16^+CD56^+$ NK cell subsets. NKT cells were analyzed within $CD3^+$ gated lymphocytes. B cells were analyzed within gated lymphocytes. Regulatory T cells ($CD4^+CD25^{high}$) were analyzed within gated $CD4^+$ T cells.

Results

Depletion of B cell ($CD19^+$) occurred in all three patients SLE and RA treated with RTX, 6 weeks after treatment (Fig. 1a). A slight increase in the percentage of circulating T ($CD3^+$) cells in all patients after RTX treatment were observed (Fig. 1b) while only a small rise in T $CD4^+$ cells in SLE2 patient was detected (Fig. 1c). A higher population of circulating regulatory $CD4^+CD25^{high}$ T cells in all treated patients was noted (Fig. 1d), and comparison between Fig. 1c, d indicates that the raised regulatory T cell population could not be attributed to a higher frequency of total T $CD4^+$ cell count after treatment.

The frequency of the total NK cells ($CD3^-CD56^{+}$), were lower in patients either before or after treatment in comparison to controls (Fig. 2a). The patient with sustained clini-

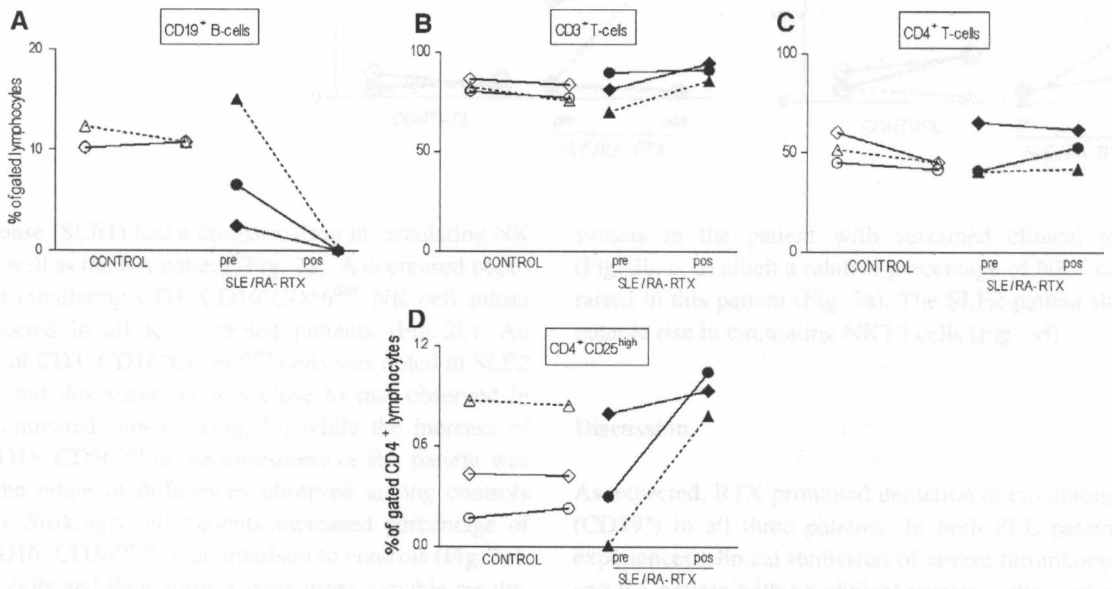

Fig. 1 Analysis of total lymphocytes T and B cell subsets and regulatory $CD4^+CD25^{high}$ T cell present in peripheral blood of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) before and after treatment with rituximab (SLE/RA-RTX). SLE1 (*filled square*), SLE2 (*filled circle*), (RA3) (*filled triangle*) and controls on left (*open symbols*). Lymphocyte phenotypic analysis was performed using a triple-labelling protocol: anti-CD3 FITC anti-CD19 PE or anti-CD4 Cy to identify **a** total B cells ($CD19^+$), **b** total T cells ($CD3^+$), (C) T cell subset ($CD4^+$) analyzed within gated lymphocytes. Double staining with anti-CD4 Cy and anti-CD25 PE, was used to identify regulatory $CD4^+CD25^{high}$ T cells (D) within gated $CD4^+$ T cells

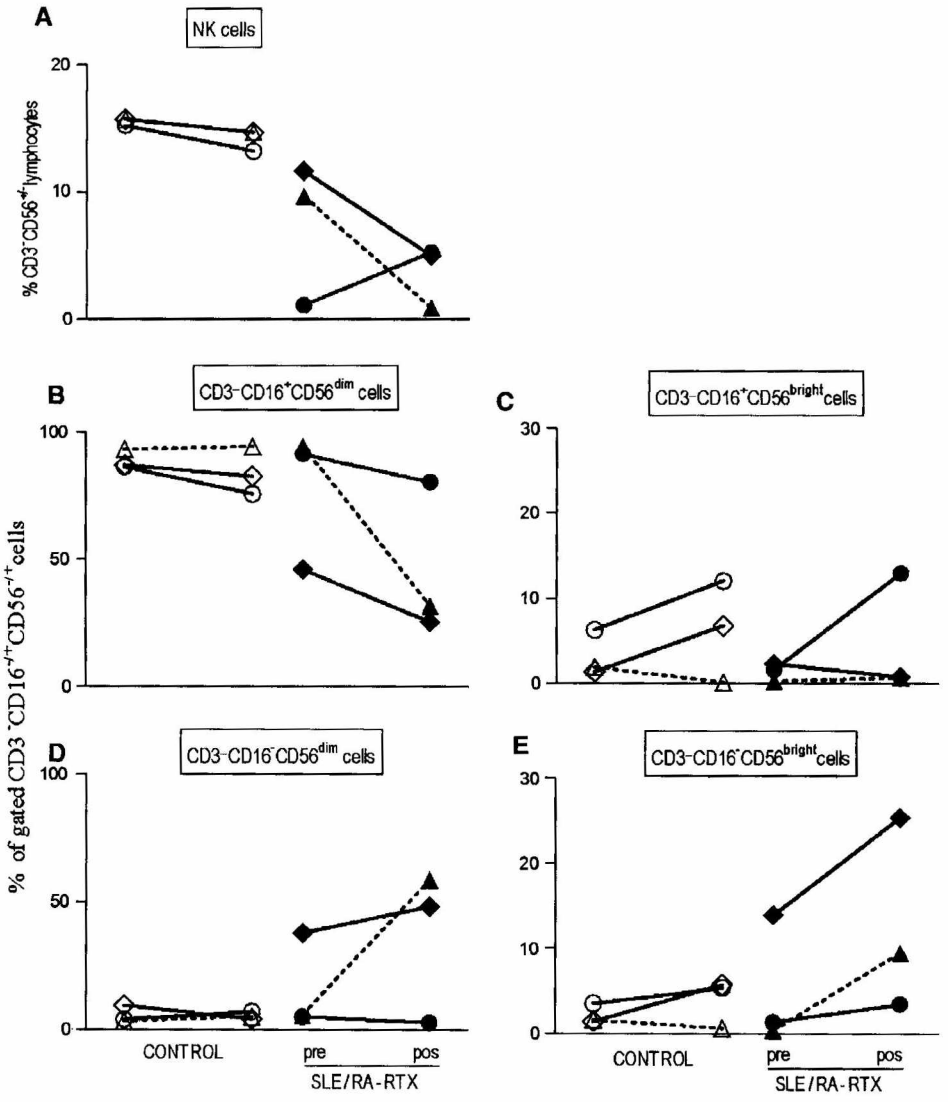

Fig. 2 Analysis of total natural killer (NK) cell and subsets present in peripheral blood of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) before and after treatment with rituximab (SLE/RA-RTX). SLE1 (*filled square*), SLE2 (*filled circle*), (RA3) (*filled triangle*) and controls on left (*open symbols*). Phenotypic studies were performed using a triple-labelling protocol anti-CD3 FITC, anti-CD56 PE and anti-CD16 PE-Cy5 to identify **a** total NK cells $CD3^-CD16^{\mp}CD56^{+}$, **b, d** $CD3^-CD16^+CD56^{dim}$ cells and $CD3^-CD16^-CD56^{dim}$ cells, **c, e** $CD3^-CD16^+CD56^{bright}$ cells and $CD3^-CD16^-CD56^{bright}$ cells. The results of total NK cells were calculated within gated lymphocytes and its subsets within NK cells

cal response (SLE1) had a dramatic drop in circulating NK cells, as well as the RA patient (Fig. 2a). A decreased population of circulating $CD3^-CD16^+CD56^{dim}$ NK cell subset was detected in all RTX treated patients (Fig. 2b). An increase of $CD3^-CD16^+CD56^{bright}$ cells was noted in SLE2 patients, but this variation was close to that observed in control untreated subjects (Fig. 3c) while the increase of $CD3^-CD16^-CD56^{dim}$ in the unresponsive RA patient was out of the range of differences observed among controls (Fig. 2d). Strikingly, all patients increased percentage of $CD3^-CD16^-CD56^{bright}$ in comparison to controls (Fig. 2e).

NKT cells and their subsets gave more variable results. The results of total NKT ($CD3^+CD56^+$) are illustrated in Fig. 3A. NKT cells analysis was classified into NKT1, $CD3^+CD16^+CD56^-$ (Fig. 3b), NKT2, $CD3^+CD16^-CD56^+$ (Fig. 3c) and NKT3 $CD3^+CD16^+CD56^+$ (Fig. 3d). The most consistent observation was a reduction of all NKT subsets in the patient with sustained clinical response (Fig. 3b, c, d) albeit a relative percentage of NKT cells was raised in this patient (Fig. 3a). The SLE2 patient showed a notable rise in circulating NKT3 cells (Fig. 3d).

Discussion

As expected, RTX promoted depletion of circulating B cell ($CD19^+$) in all three patients. In both SLE patients who experienced clinical remission of severe thrombocytopenia and RA patient with no clinical response, the expression of $CD19^+$ B cells in peripheral leukocytes was undetectable 6 weeks after treatment (Fig. 1a). Although some authors observed a direct relationship between decreased $CD19^+$ B cell and clinical improvement in SLE [2, 19], not all medical literature on RTX therapy supports this concept [1]

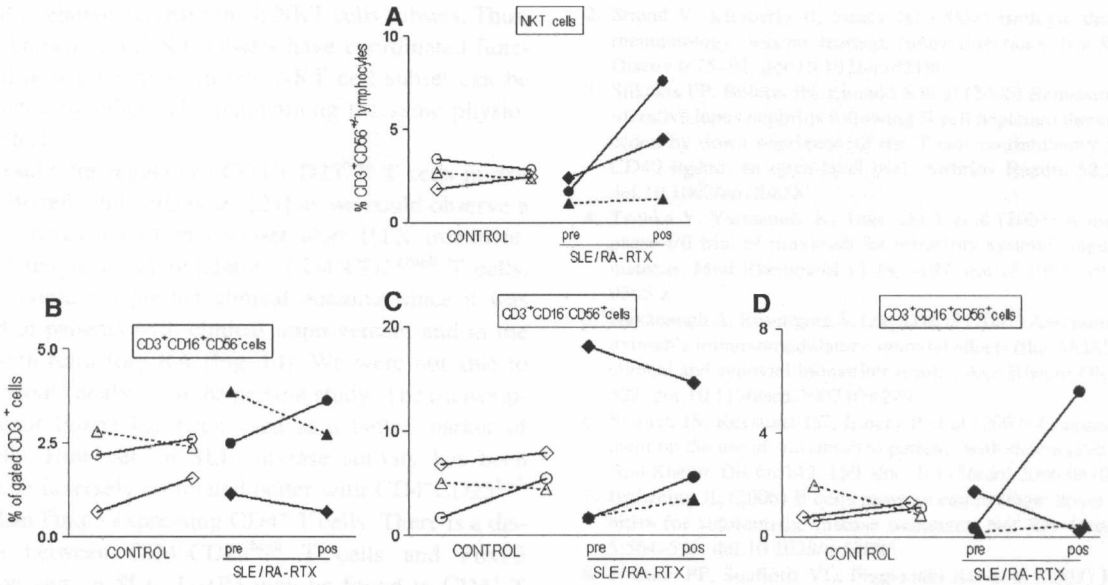

Fig. 3 Analysis of total natural killer T (NKT) cells and subsets present in peripheral blood of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) before and after treatment with rituximab (SLE/RA-RTX). SLE1 (*filled square*), SLE2 (*filled circle*), (RA3) (*filled triangle*) and controls on left (*open symbols*). NKT phenotypic analysis was performed using a triple-labelling protocol anti-CD3 FITC, anti-CD56 PE and anti-CD16 PE-Cy5 to identify **a** total NKT cells ($CD3^+CD16^{\mp}CD56^{\pm}$), **b** NKT1 cells ($CD3^+CD16^+CD56^-$/$CD3^+$), **c** NKT2 cells ($CD3^+CD16^-CD56^+$/$CD3^+$) and **d** NKT3 cells ($CD3^+CD16^+CD56^+CD3^+$) analyzed within gated $CD3^+$ lymphocytes

suggesting that other not evaluated immune factors may be involved. Indeed, although the B cells depletion occurred after RTX therapy in all three patients of the present study, the clinical response was variable. Both SLE patients present severe thrombocytopenia as the major clinical complication, a situation in which autoantibodies are clearly pathogenic [20].

In this study, we investigated NK cells subsets based on the cell surface of NK cells expressing lower level of CD56 ($CD56^{dim}CD16^+$), known as a more cytotoxic subset, whereas NK cells ($CD56^{bright}CD16^{\mp}$) are involved in the regulation, based on the observation that $CD56^{bright}$ subset is the major source of IFN-γ cytokines [21]. RTX and other anti-CD20 monoclonal antibodies are known to promote downregulation of CD16, which is the IgG receptor they use to mediate their effector functions [22]. Therefore, it was important to verify NK cells subsets according to CD16 expression (Fig. 2). All patients had a relative lower population of circulating NK cells when compared to control subjects. The patient with sustained clinical response had a notable decrease of total NK cell population in peripheral blood, but so did the patient with non responsive RA (Fig. 2a). The most striking observation was the rise in circulating $CD56^{bright}$ $CD16^-$ (regulatory NK cells) in the patient with sustained clinical response. More importantly, this elevation occurred in that patient who had higher baseline population of circulating regulatory NK cells. This observation encourages future works focusing on evaluation of regulatory NK cells as a prognostic factor that may predict clinical response after RTX therapy [that could be obtained as early as 6 months (weeks) after initiation of treatment]. In addition, baseline circulating regulatory NK cells must also be explored as a predictor of clinical response. In Fig. 2, we also show that all patients experienced a decrease in $CD3^-CD16^+CD56^{dim}$ cells (Fig. 2b) and a rise in $CD3^-CD16^-CD56^{bright}$ (Fig. 2e). Although these data may suggest a switch from more cytotoxic population to regulatory NK cells, it should be highlighted that such changes may be partially or entirely explained by the downregulation of CD16 promoted by anti-CD20 therapies [22].

NKT cells were further characterized accordingly to the previous reported classification by Vitelli-Avelar and colleagues [18]: NKT1 cells ($CD3^+CD16^+CD56^-$), NKT2 cells ($CD3^+CD16^-CD56^+$) and NKT3 cells ($CD3^+CD16^+CD56^+$). They characterized subpopulations of circulating NKT cells observing increased values of NKT2 ($CD3^+CD16^-CD56^+$) cells in indeterminate forms of Chagas disease thus suggesting a potential role of these cells in immunomodulation that may prevent progression to chronic cardiac disease. In the present study, no specific change in NKT subsets could be associated with successful treatment (Fig. 3b–d). An increasing population of circulating NKT3 subset outside the range of variation among controls and other patients was observed in patient SLE2. Curiously, in the SLE with sustained response to RTX,

there was a relative decrease in all NKT cells subsets. Thus, it is not known how NKT subsets have coordinated functions and how a decrease in one NKT cell subset can be compensated by other cells maintaining the same physiological effects.

Our results for regulatory $CD4^+CD25^{high}$ T cells mirror those of Bonelli and colleagues [23] as we could observe a trend for increasing of this subset after RTX treatment. Increased frequency of regulatory $CD4^+CD25^{high}$ T cells, however, could not predict clinical outcome since it was observed in patients with clinical improvement and in the patient with refractory RA (Fig. 1d). We were not able to perform FoxP3 analyses in the present study. The transcriptional factor Foxp3 has been used as a better marker of these cells. However, in SLE, disease activity has been found to be inversely correlated better with $CD4^+CD25^{high}$ T cells than Foxp3 expressing $CD4^+$ T cells. There is a dissociation between $CD4^+CD25^{high}$ T cells and FoxP3 expression and, in SLE, FoxP3 may be found in $CD4^+$ T cells with low or intermediate expression of CD25 suggesting it may be at least in part a marker of T cell activation [23]. In contrast, FoxP3 has been suggested by other recent papers to be a strong biomarker of response after RTX therapy [7, 24].

Overall, our results indicate that measurement of circulating B and T lymphocytes, NK and NKT cells subsets, are subject to considerable variations after RTX therapy. It is not currently known to which extent such changes can be attributed to direct effects of B cell drop or as secondary compensatory mechanism. For instance, macrophage phenotypical changes after RTX infusion are interpreted as a compensatory response to loss of APC function of B cells [9], while reduced markers of T cell activation are considered a direct response of B cell drop and loss of a source of antigen presentation and cytokines production [3, 8]. Future works on the role of regulatory NK and NKT cells subsets after RTX therapy should have special focus on the association between regulatory $CD56^{bright}$ NK cell subset and decreased of NKT cell subsets with sustained clinical response.

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Conflict of interest statement The authors have no conflict of interest.

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