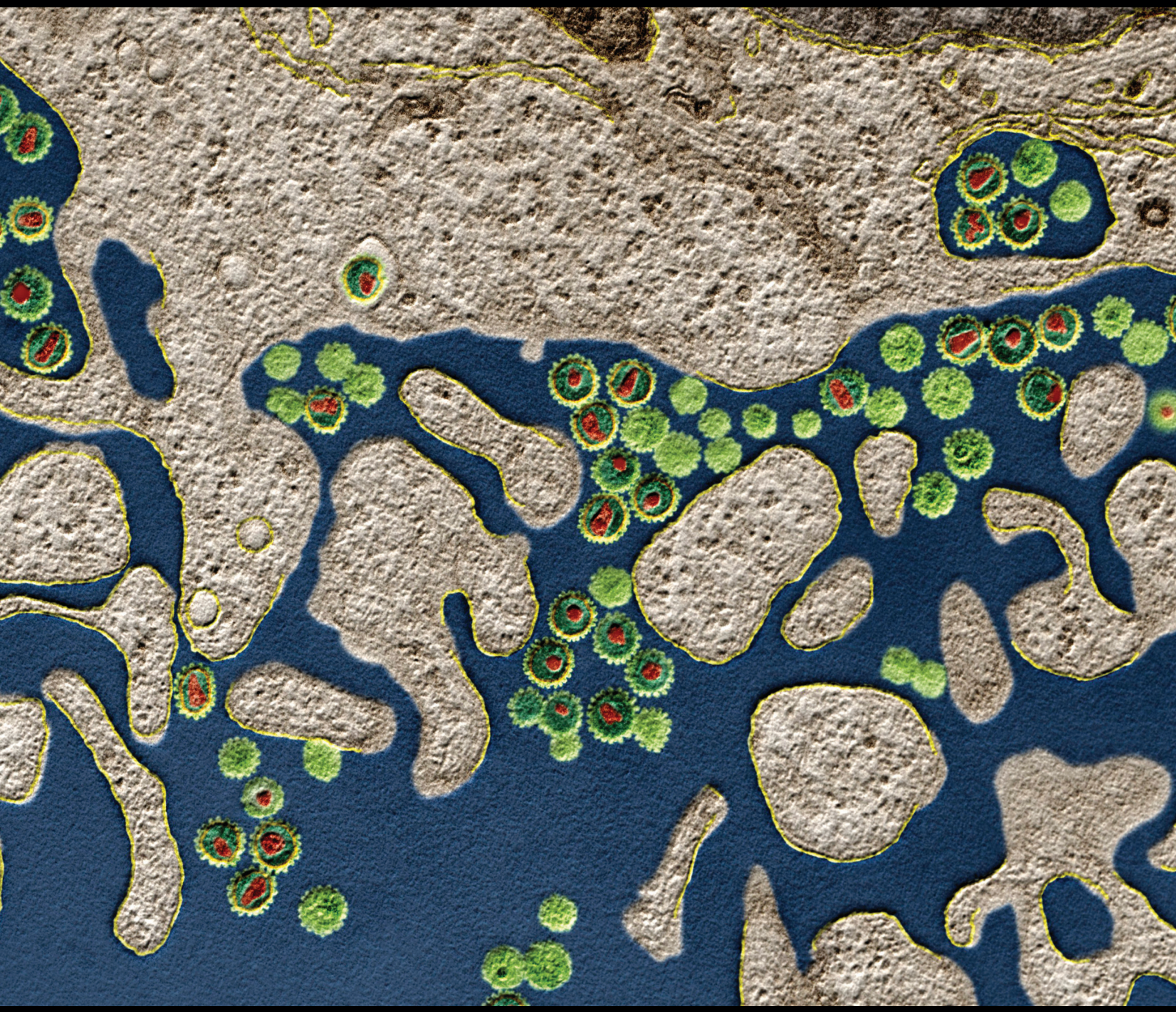
Journal of Immunology Research

Systemic Lupus Erythematosus and Rheumatoid Arthritis

Guest Editors: Xuan Zhang, Lingyun Sun, Thomas Dörner, and Peter E. Lipsky



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Editorial Board

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 814748, 5 pages
http://dx.doi.org/10.1155/2015/814748

Research Article

High Avidity dsDNA Autoantibodies in Brazilian Women with Systemic Lupus Erythematosus: Correlation with Active Disease and Renal Dysfunction

Rodrigo C. Oliveira,[1] Isabela S. Oliveira,[1] Mittermayer B. Santiago,[2] Maria L. B. Sousa Atta,[3] and Ajax M. Atta[3]

[1]*Programa de Pós-Graduação em Imunologia, Instituto de Ciências da Saúde, Universidade Federal da Bahia, 40140-100 Salvador, BA, Brazil*
[2]*Escola Bahiana de Medicina e Saúde Pública, 40050-420 Salvador, BA, Brazil*
[3]*Laboratório de Pesquisa em Imunologia, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal da Bahia, Rua Barão de Jeremoabo 147, 40170-115 Salvador, BA, Brazil*

Correspondence should be addressed to Ajax M. Atta; ajatta@ig.com.br

Received 21 November 2014; Accepted 16 March 2015

Academic Editor: Thomas Dörner

We investigated in Brazilian women with SLE the prevalence and levels of high avidity (HA) dsDNA antibodies and tested their correlation with lupus activity and biomarkers of renal disease. We also compared these correlations to those observed with total dsDNA antibodies and antibodies against nucleosome (ANuA). Autoantibodies were detected by ELISA, while C3 and C4 levels were determined by nephelometry. Urine protein/creatinine ratio was determined, and lupus activity was measured by SLEDAI-2K. The prevalence of total and HA dsDNA antibodies was similar to but lower than that verified for ANuA. The levels of the three types of antibodies were correlated, but the correlation was more significant between HA dsDNA antibodies and ANuA. High avidity dsDNA antibodies correlated positively with ESR and SLEDAI and inversely with C3 and C4. Similar correlations were observed for ANuA levels, whereas total dsDNA antibodies only correlated with SLEDAI and C3. The levels of HA dsDNA antibodies were higher in patients with proteinuria, but their levels of total dsDNA antibodies and ANuA were unaltered. High avidity dsDNA antibodies can be found in high prevalence in Brazilian women with SLE and are important biomarkers of active disease and kidney dysfunction.

1. Introduction

In lupus, there is an important autoreactivity of B lymphocytes shown by the production of more than 160 specificities of autoantibodies and circulating immune complexes of autoantibodies and autoantigens [1–3]. The dsDNA autoantibody is the most important laboratory biomarker of SLE associated with both disease activity and renal dysfunction. However, the autoantibody's involvement in lupus immunopathogenesis still deserves more investigation [4–6]. Although this antibody shows high SLE specificity, its prevalence in different studies has been estimated to be around 50% [7, 8]. In addition, dsDNA antibodies can be found in patients regardless of whether they have renal disease. Interestingly, dsDNA antibodies exhibit a high degree of heterogeneity, as shown by their cross-reactions with other autoantigens and different isotypes as well as by changes in their affinity to bind dsDNA epitopes [9–11]. This study investigated the prevalence of dsDNA autoantibodies of high avidity and their correlations with clinical and laboratory findings in SLE patients living in northeastern Brazil. In addition, these correlations were compared to those obtained with total dsDNA antibodies and nucleosome antibodies.

2. Material and Methods

2.1. Patients. One hundred forty-two SLE female patients from the Rheumatology Service of the Santa Izabel Hospital

(Salvador, Bahia) were consecutively enrolled in this study. All had a previous diagnosis of lupus and exhibited four or more criteria for SLE [12]. Lupus activity was scored with the SLEDAI-2K [13]. Prednisone was the main medication used by the patients (132/142, 93.0%), combined with Chloroquine or Chloroquine plus Azathioprine (89/132, 67.4%). Thirty-two patients (32/132, 24.2%) were taking Methotrexate plus Prednisone and Chloroquine, combined or not with Azathioprine. Cyclosporine was rarely used (7/132, 5.3%). All patients signed an informed consent form to participate in this study, which was approved by the Ethics Committee of the Santa Izabel Hospital.

2.2. Laboratory Investigation. Anti-dsDNA IgG antibodies were first tested by the indirect fluorescent antibody test with *Crithidia luciliae* (CLIFT), followed by an indirect ELISA to measure their serum levels (Orgentec Diagnostika GmbH, Germany). Afterward, the presence and levels of high avidity dsDNA IgG antibodies were measured with the QUANTA Lite test HA dsDNA ELISA (INOVA Diagnostics Inc., San Diego, CA, USA). The cutoffs in the ELISA test were 25 IU/mL and 30 IU/mL, respectively. An indirect ELISA, using a cutoff of 20 U/mL (Orgentec), determined the levels of nucleosome antibodies. Cellular analysis of blood was done with the cytometer CellDyn-Ruby (Abbot Diagnostic Inc., USA) while inflammation was measured by erythrocyte sedimentation rate. Serum levels of complement C3 (reference range = 67–149 mg/dL) and C4 (reference range = 10–38 mg/dL) were determined by nephelometry in the Image Immunochemistry System (Beckman-Coulter, USA). In addition, the presence of renal dysfunction was obtained by chemical and microscopic examination of fresh urine using the analyzer LabUMat UriSed (Electronic Muszeripari Kft, Budapest). Colorimetric methods measured the levels of urine protein and urine creatinine. In this study, a significant proteinuria was a urine protein/creatinine ratio (P/C ratio) >0.23. This cutoff was calculated with a receiver operating characteristics (ROC) curve using the P/C ratios of patients having negative or positive diagnosis of lupus nephritis when they were included in the study (cutoff = 0.23, AUC = 0.904; sensitivity = 88.5%, specificity = 80.3%).

2.3. Statistical Analysis. The test of D'Agostino and Pearson analyzed the distribution of the continuous variables, which were presented as mean ± SD or median and interquartile range (IQR, 25–75%). The test of Spearman performed correlation analyses, which were validated for their statistical significance in accordance with the number of *XY* pairs tested. The means and medians of two groups were compared with the unpaired *t*-test and *U* test of Mann-Whitney, respectively. The significance level was significant at $P < 0.050$. The statistical software GraphPad 6.0 and MedCalc 13.0 were used.

3. Results

3.1. Clinical and Demographic Data. Lupus patients had a mean age of 40.6 ± 12.9 years (95% CI = 38.5–42.7 years), ranging from 17 to 79 years. The median of lupus duration in these women was eight years, varying from 0.4 to 40 years. One hundred twenty-five patients (88.0%) had active lupus. In 80/142 (56.3%) patients, the activity varied from moderate to very high (median = 9, range 6–31). Sixty patients had a clinical diagnosis of kidney disorder demonstrated by proteinuria and presence of urine leukocytes, erythrocytes, and less frequently urinary casts [14]. Autoantibodies and low C3 and C4 levels were found in different prevalence in the patients, predominating ANA, AnuA, and dsDNA antibodies (Table 1).

Table 1: Immunological findings in Brazilian SLE women.

Immune marker	Prevalence *N* 142 (%)	Level (median, IQR)
ANA	131 (92.2)	320 (160–1,280)
ANuA (U/mL)	118 (83.1)	114 (44–196)
Total dsDNA Ab (IU/mL)	72 (50.7)	205 (78–279)
HA dsDNA Ab (IU/mL)	66 (46.5)	189 (95–525)
Sm Ab (U/mL)	35 (24.6)	72 (51–335)
RNP-70 Ab (U/mL)	48 (33.8)	106 (91–244)
SS-A/Ro Ab (U/mL)	55 (38.7)	87 (56–221)
SS-B/La Ab (U/mL)	13 (9.1)	101 (45–304)
Rib-P Ab (U/mL)	20 (14.1)	18 (16–190)
IgA anti-B2GPI (U/mL)	32 (22.5)	26 (19–41)
IgG anti-B2GPI (U/mL)	16 (11.3)	20 (12–34)
IgM anti-B2GPI (U/mL)	9 (6.3)	22 (19–53)
IgA aCL (U/mL)	3 (<5.0)	38 (15–40)
IgG aCL (U/mL)	11 (7.7)	18 (10–37)
IgM aCL (U/mL)	7 (<5.0)	36 (26–65)
C3 low (<67 mg/mL)	36 (25.3)	57 (41–64)
C4 low (<10 mg/mL)	29 (20.4)	5 (4–8)

3.2. Correlation Analysis. There was a correlation between the levels of total dsDNA antibodies and HA dsDNA antibodies (*XY* pairs = 66, $r = 0.50$; $P < 0.0001$). On the other hand, the levels of total dsDNA antibodies and of HA dsDNA antibodies were correlated with ANuA levels (*XY* pairs = 70, $r = 0.34$; $P = 0.004$ and *XY* pairs = 65, $r = 0.61$, $P < 0.0001$, resp.). However, the correlation between HA dsDNA antibodies and nucleosome antibodies was higher ($P = 0.044$). The levels of total dsDNA antibodies were only correlated with SLEDAI scores and C3 levels. Differently, the levels of both HA dsDNA antibodies and ANuA, besides correlating with SLEDAI scores and the C3 levels, were also correlated with C4 levels and ESR (Table 2).

Table 2: Correlation of total dsDNA antibodies, HA dsDNA antibodies, and ANuA with clinical and laboratory findings in SLE patients.

	Total dsDNA Ab	HA dsDNA Ab	ANuA
SLEDAI	0.33 0.005	0.45 0.0001	0.43 <0.0001
ESR	0.29 NS	0.36 0.003	0.37 <0.0001
C3	−0.34 0.003	−0.55 <0.0001	−0.32 <0.001
C4	−0.24 NS	−0.34 0.006	−0.28 0.002
P/C ratio	0.12 NS	0.24 NS	0.24 NS

Total dsDNA antibody, *XY* pairs = 72; HA dsDNA antibody, *XY* pairs = 66; ANuA, *XY* pairs = 118. NS: not significant.

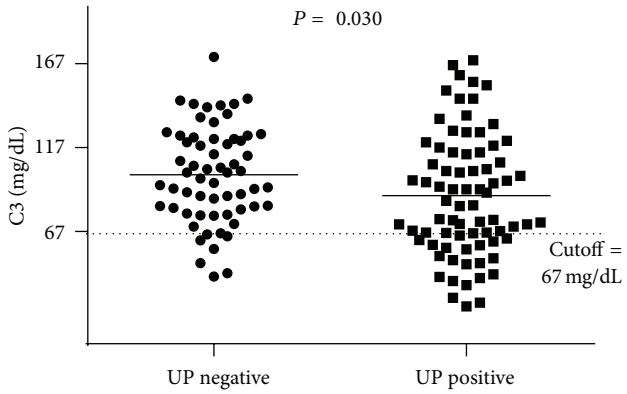

Figure 1: Serum levels of C3 in SLE patients without proteinuria (UP negative, P/C ratio ≤ 0.23) and presenting urine protein (UP positive, P/C ratio > 0.23).

3.3. Proteinuria, C3, and Autoantibody Levels. The serum levels of C3 were lower in the patients with a P/C ratio >0.23 (Figure 1). There was a difference between the levels of HA dsDNA antibodies in patients with and without proteinuria (P/C ratio > 0.23; $P = 0.037$). However, the levels of total dsDNA antibodies and ANuA were similar in these two groups of patients ($P = 0.571$ and $P = 0.065$, resp.) (Figure 2).

4. Discussion

Anti-dsDNA IgG autoantibodies are important biomarkers in systemic lupus erythematosus. Nevertheless, the Farr RIA or another immunoassay to detect high avidity dsDNA antibodies is not routinely used in the rheumatology laboratory, being widely substituted by ELISA tests that measure total dsDNA antibody levels. These immunoassays do not discriminate between antibodies of low and high affinity or antibodies that cross-react with dsDNA epitopes. In contrast, ELISA tests that detect HA dsDNA are comparable to Farr RIA [10, 15–19]. Although dsDNA antibodies have been associated with lupus activity and lupus nephritis, the role of these antibodies in SLE pathogenesis still deserves more study. To date, renal disease has been demonstrated in a large proportion of SLE patients who are seronegative for dsDNA antibodies and can be absent in patients who have high levels of these autoantibodies.

Previously, we did not find an association between laboratory findings of lupus kidney disease such as proteinuria and altered urine exam in Brazilian patients with high levels of total dsDNA antibodies. Such observation suggested the need of more studies to characterize the avidity of these autoantibodies [20]. In the present study, nucleosome antibodies were correlated with total dsDNA antibodies and more strongly with HA dsDNA antibodies. This finding was expected because nucleosome is a molecular complex constituted by histones, nonhistone proteins, and dsDNA. Thus, the presence of dsDNA epitopes in nucleosome can elicit specific autoantibodies that also participate in the immune reactions of tests that detect dsDNA antibodies, mainly of high avidity, justifying these correlations.

Herein, we demonstrated that total dsDNA antibodies measured by a routine indirect ELISA can present a correlation with lupus activity and C3 levels. However, the levels of HA dsDNA antibodies and ANuA, besides exhibiting good correlation with SLEDAI and C3 levels, were also correlated with low C4 levels and ESR. In lupus, immune complexes formed by IgG and IgM autoantibodies and self-antigens activate complement lowering both C3 and C4 levels. Both low C3 and C4 are biomarkers of disease activity and were recently included as immunologic criteria for SLE by the Systemic Lupus International Collaborating Clinics (SLICC) group [21]. Together with dsDNA antibodies, low C3 and C4 are also biomarkers of lupus nephritis, but low C3 levels seem to be more sensitive than low C4 levels to diagnose renal SLE flares. In the present work, C3 levels were more strongly correlated with the levels of HA dsDNA antibodies, being that this correlation was higher than that of C4 levels. In contrast with C4 levels, C3 levels were lower in the patients with renal disorder. Interestingly, only the levels of HA dsDNA antibodies were higher in SLE patients with proteinuria, here demonstrated by a urine P/C ratio above 0.23. Compared with 24 h urine protein, the use of spot urine P/C ratio still is controversial. However, several studies have supported the use of P/C ratio in the clinical practice, and it has been recently adopted by the SLICC study [21]. The findings presented here do not exclude the participation of other immune mediators in the pathogenesis of kidney disease in these individuals. Thus, the contribution of C1q antibodies, as well as the involvement of different isotypes of dsDNA antibodies, activated T lymphocytes, or inflammatory cytokines, must also be considered [22–24].

In conclusion, HA dsDNA antibodies can be found with high prevalence in Brazilian women with SLE and seem to be important biomarkers of active disease and contribute to kidney dysfunction in these patients.

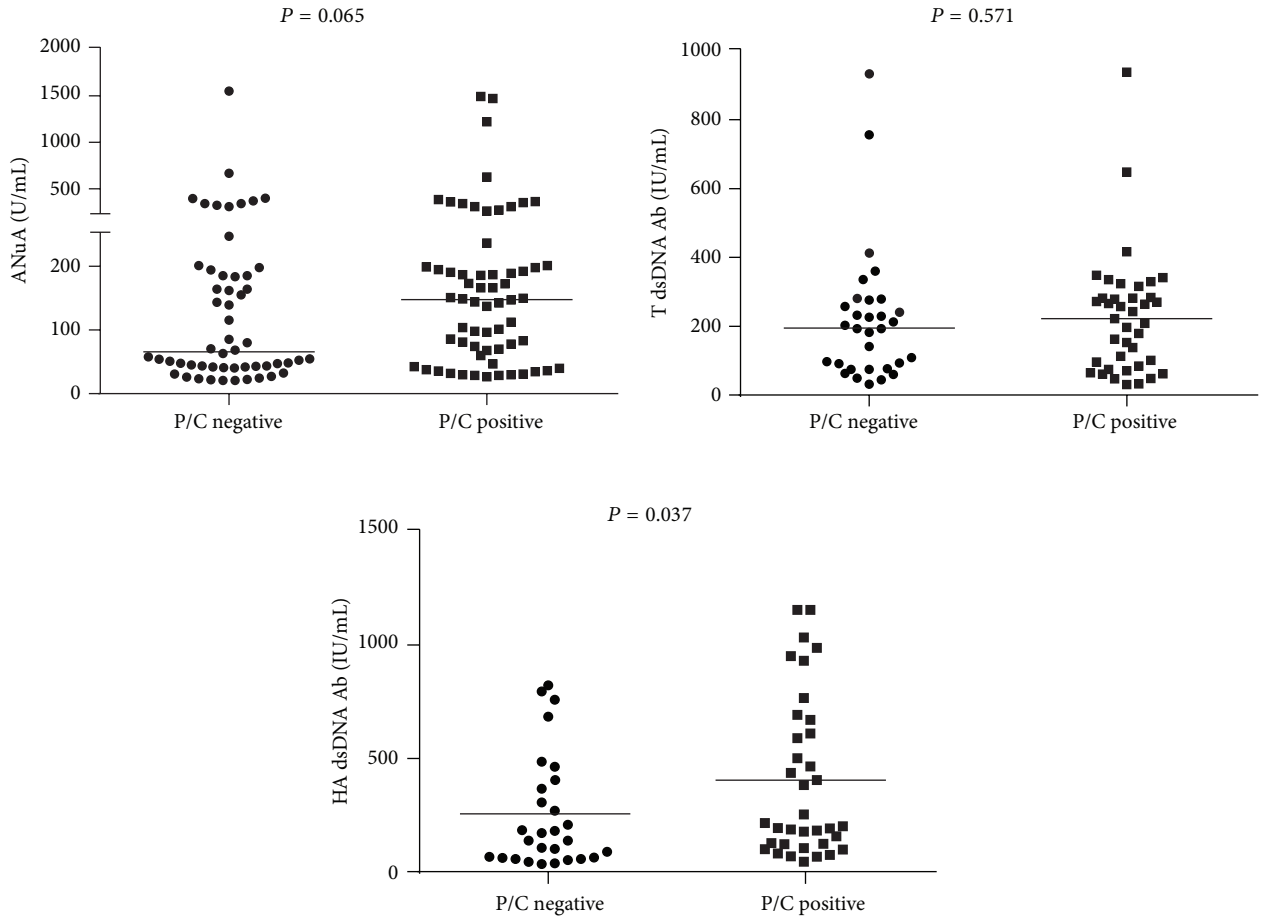

Figure 2: Levels of total dsDNA antibodies, HA dsDNA antibodies, and ANuA in SLE Brazilian women without and with proteinuria (P/C ratio ≤ 0.23 and >0.23, resp.). The medians are represented by horizontal lines and were compared with the *U* test of Mann-Whitney.

Conflict of Interests

The authors have no conflict of interests that is directly relevant to the content of this paper.

Acknowledgments

Research is supported by the National Council for Scientific and Technological Development (CNPq), Grant 306.097/2010-9. Ajax M. Atta, Maria L. B. Sousa Atta, and Mittermayer B. Santiago are recipients of CNPq-BPQ fellowships. Isabela S. Oliveira is a recipient of a CAPES scholarship and Rodrigo C. Oliveira is recipient of a FAPESB scholarship.

References

[1] C. C. Mok and C. S. Lau, "Pathogenesis of systemic lupus erythematosus," *Journal of Clinical Pathology*, vol. 56, no. 7, pp. 481–490, 2003.

[2] C. K. Li and D. A. Isenberg, "Systemic lupus erythematosus," *Medicine*, vol. 34, no. 11, pp. 445–452, 2006.

[3] J. J. Manson and A. Rahman, "Systemic lupus erythematosus," *Orphanet Journal of Rare Diseases*, vol. 1, no. 1, article 6, 2006.

[4] J. H. M. Berden, "Lupus nephritis," *Kidney International*, vol. 52, no. 2, pp. 538–558, 1997.

[5] J. Cortés-Hernández, J. Ordi-Ros, M. Labrador et al., "Anti-histone and anti-double-stranded deoxyribonucleic acid antibodies are associated with renal disease in systemic lupus erythematosus," *The American Journal of Medicine*, vol. 116, no. 3, pp. 165–173, 2004.

[6] M. Sui, Q. Lin, Z. Xu et al., "Simultaneous positivity for anti-DNA, anti-nucleosome and anti-histone antibodies is a marker for more severe lupus nephritis," *Journal of Clinical Immunology*, vol. 33, no. 2, pp. 378–387, 2013.

[7] H.-A. Kim, J.-Y. Jeon, G.-S. Choi et al., "The antichromatin antibodies can be useful as a diagnostic tool and disease activity marker of systemic lupus erythematosus in Koreans," *Clinical Immunology*, vol. 128, no. 2, pp. 277–283, 2008.

[8] A. M. Atta, M. B. Santiago, F. G. Guerra, M. M. Pereira, and M. L. B. Sousa Atta, "Autoimmune response of IgE antibodies to cellular self-antigens in systemic lupus erythematosus," *International Archives of Allergy and Immunology*, vol. 152, no. 4, pp. 401–406, 2010.

[9] A. Doria and M. Gatto, "Nephritogenic-antinephritogenic antibody network in lupus glomerulonephritis," *Lupus*, vol. 21, no. 14, pp. 1492–1496, 2012.

[10] D. Villalta, P. B. Romelli, C. Savina et al., "Anti-dsDNA antibody avidity determination by a simple reliable ELISA method for

SLE diagnosis and monitoring," *Lupus*, vol. 12, no. 1, pp. 31–36, 2003.

[11] U. Heidenreich, G. Mayer, M. Herold, W. Klotz, K. S. Al-Jazrawi, and K. Lhotta, "Sensitivity and specificity of autoantibody tests in the differential diagnosis of lupus nephritis," *Lupus*, vol. 18, no. 14, pp. 1276–1280, 2009.

[12] M. C. Hochberg, "Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 40, no. 9, p. 1725, 1997.

[13] Z. Touma, M. B. Urowitz, and D. D. Gladman, "SLEDAI-2K for a 30-day window," *Lupus*, vol. 19, no. 1, pp. 49–50, 2010.

[14] M. A. Dooley, C. Aranow, and E. M. Ginzler, "Review of ACR renal criteria in systemic lupus erythematosus," *Lupus*, vol. 13, no. 11, pp. 857–860, 2004.

[15] R. J. T. Smeenk, "Detection of antibodies to dsDNA: current insights into its relevance," *Clinical & Experimental Rheumatology*, vol. 20, no. 3, pp. 294–300, 2002.

[16] A. Rahman and F. Hiepe, "Anti-DNA antibodies—overview of assays and clinical correlations," *Lupus*, vol. 11, no. 12, pp. 770–773, 2002.

[17] D. Isenberg and R. Smeenk, "Clinical laboratory assays for measuring anti-dsDNA antibodies. Where are we now?" *Lupus*, vol. 11, no. 12, pp. 797–800, 2002.

[18] A. G. Tzioufas, C. Terzoglou, E. D. Stavropoulos, S. Athanasiadou, and H. M. Moutsopoulos, "Determination of anti-dsDNA antibodies by three different methods: comparison of sensitivity, specificity and correlation with lupus activity index (LAI)," *Clinical Rheumatology*, vol. 9, no. 2, pp. 186–192, 1990.

[19] B. B. Suh-Lailam, T. R. Chiaro, K. W. Davis, A. R. Wilson, and A. E. Tebo, "Evaluation of a high avidity anti-dsDNA IgG enzyme-linked immunosorbent assay for the diagnosis of systemic lupus Erythematosus," *International Journal of Clinical and Experimental Pathology*, vol. 4, no. 8, pp. 748–754, 2011.

[20] A. M. Atta, M. M. Pereira, M. Santiago, and M. L. B. Sousa-Atta, "Anti-dsDNA antibodies in Brazilian patients of mainly African descent with systemic lupus erythematosus: lack of association with lupus nephritis," *Clinical Rheumatology*, vol. 28, no. 6, pp. 693–697, 2009.

[21] M. Petri, A.-M. Orbai, G. S. Alarcõn et al., "Derivation and validation of the systemic lupus international collaborating clinics classification criteria for systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 64, no. 8, pp. 2677–2686, 2012.

[22] A. M. Orbai, L. Truedsson, G. Sturfelt et al., "Anti-C1q antibodies in systemic lupus erythematosus," *Lupus*, vol. 24, no. 1, pp. 42–49, 2015.

[23] R. Saxena, T. Mahajan, and C. Mohan, "Lupus nephritis: current update," *Arthritis Research and Therapy*, vol. 13, no. 5, article 240, 2011.

[24] B. Dema, C. Pellefigues, S. Hasni et al., "Autoreactive IgE is prevalent in systemic lupus erythematosus and is associated with increased disease activity and nephritis," *PLoS ONE*, vol. 9, no. 2, Article ID e90424, 2014.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 238263, 5 pages
http://dx.doi.org/10.1155/2015/238263

Review Article

Peer Support and Psychosocial Pain Management Strategies for Children with Systemic Lupus Erythematosus

Laura Nabors, Teminijesu John Ige, and Bradley Fevrier

Health Promotion and Education Program, School of Human Services, University of Cincinnati, Cincinnati, OH 45221-0068, USA

Correspondence should be addressed to Laura Nabors; naborsla@ucmail.uc.edu

Received 30 March 2015; Accepted 25 May 2015

Academic Editor: Lingyun Sun

This paper reviews information on Systemic Lupus Erythematosus (SLE) in children. Children with this chronic illness often experience pain related to their condition. They also can experience social isolation. This paper reviews psychosocial information on peer support and cognitive behavioral pain management strategies. The information presented in this paper provides new insights for health professionals assisting children and families in coping with psychological facets of this disease. Research focusing on ways by which peers and friends can support the child's use of psychological pain management strategies will provide new information for the literature.

1. Introduction

Children with Systemic Lupus Erythematosus (SLE) may experience chronic pain, cognitive dysfunction, and dermatological problems. SLE manifests in complex ways in children and can involve multiple organs [1]. There are many sources of pain for children with SLE, but some common ones are related to renal problems, abdominal pain, chest pain, and headaches [2]. SLE can impact the central nervous system, resulting in headaches, seizures, and cerebrovascular disease, all of which can result in increased pain experiences for children. Children with SLE may experience significant pain that goes unmanaged. In addition to physical complications, children with SLE can face a host of psychosocial issues. For example, children with SLE may experience depression and become isolated, which may have significant adverse effects on a child's interpersonal relationships [2–4]. The current paper reviews literature on peer relationships and psychosocial/cognitive behavioral interventions for pain management. Information presented in this paper allows health care professionals to view two key issues for children with SLE.

2. Prevalence and Incidence of SLE in Children

Variations exist in estimates of the incidence and prevalence of SLE [5, 6]. SLE is less common among children, as it is most often diagnosed in reproductive age in women. Approximately 0.36 to 1 per 100,000 children have SLE. This disease is more common in females and more severe for those in minority groups. SLE in children may be related to depression and other psychological symptoms that reduce quality of life [7–9].

3. Peer Relationships of Children with SLE

Peer relationships are vital in building self-esteem and a sense of acceptance and belonging, which are traits required for optimal psychosocial functioning [3, 10]. Peer support in healthcare can be defined as the provision of informational and emotional support by a peer who has acquired knowledge through experience and who possesses similar characteristics as the ill person [11]. Peer relationships are vital as children transition into adolescence where their sense of self determines how they gain independence, assume responsibility, and form successful interpersonal relationships [12]. Peer support can help to increase optimism and alleviate feelings of isolation and loneliness. This may assist children to better understand their illness and to learn and adopt healthy behaviors and disease management skills [11].

Children with SLE can experience challenges in building peer relationships due to experiencing isolation, low self-esteem, and coping with neurocognitive and affective disorders [2, 3]. Isolation that occurs within the context of chronic illness may lead to fewer friendships and lower levels of involvement in activities with peers [4, 13]. This situation may, in some cases, set the stage for social difficulties in adolescence and adulthood [14]. There is little information about interventions to improve peer support for children with SLE and little information about whether those with higher levels of peer support experience better functioning, reduced pain, and improved quality of life.

Research assessing peer support of children with chronic illnesses has shown that peer support can improve children's attitudes and functioning. For instance, in a randomized controlled trial of a "peer support" intervention for children with chronic illnesses, Rhee et al. found significant improvements in positive attitudes toward illness in an intervention group [15]. Also, McLaughlin et al. discovered that participating in a peer support social networking site led to improved social bonding in chronically ill children [16]. In their study, children who reported lacking support from others, poor family interactions, and low self-efficacy were more likely to use the social networking site. Consequently, when other types of support are low, social support from peers may be a resilience factor. In another study, Al-Sheyab and colleagues discovered significant improvements among participants of a peer-led education and support group in three areas: health-related quality of life, self-efficacy to resist negative health behaviors, and knowledge of best practices for illness management [17].

Health care professionals should inquire about social functioning for children with SLE and determine if a lack thereof is having a negative impact on emotional functioning and quality of life. In cases where peer support is low, several interventions may be considered. An intervention at the school level is to explain the child's medical condition to other children in the class. It is important to explain pain flares, missed school due to medical appointments, and differences in the child's behaviors while at school (e.g., riding the elevator to reduce walking) to peers in the child's class. When children have a better understanding of the nature of an illness it may be easier for them to respond appropriately to the needs of a classmate with SLE. If explaining to all peers in the classroom is not feasible, holding a meeting with the child's teacher, to explain how to educate peers on a more individual basis, may be appropriate. Involvement in extracurricular activities may afford opportunities to build social relationships. Requesting that parents monitor opportunities to engage in activities and build social opportunities is another important intervention if the child is coping with isolation or problems with emotional functioning. Finally, peers may not understand that the child with SLE has to cope with pain, take medicine, and use different pain management strategies. Carefully explaining pain flares and teaching peers about pain management may assist peers in understanding the illness and reduce any discomfort they have when interacting with the child.

4. Pain and Psychosocial Interventions for Pain for Children with SLE

Children with SLE often experience chronic pain and stiffness in joints, similar to that experienced by children with juvenile idiopathic arthritis. Experts in the field have cited pain amplification as a primary issue for children with SLE [2]. Medical treatments for joint pain include hydroxychloroquine and chloroquine. These medications reduce pain with relatively few adverse side effects. Methotrexate may be even more successful but produce greater side effects. Nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are frontline treatments for joint pain. Mild exercise and omega-3 fatty acids are other possible treatments to relieve pain and fatigue [18].

Psychological strategies to manage pain may be useful as adjunctive interventions for treatment of SLE-related pain. This research team believes that many interventions used with adults can be applied to assist children and their families. Readers interested in learning more about psychosocial interventions are directed to a recent article by Barlow and her associates [19]. This paper presents a review of psychosocial strategies to assist adults. Before teaching the child pain management strategies, it may be advantageous to explain how pain works in the body using developmentally appropriate language. Emphasizing that pain is interpreted in one's brain and that how one thinks and feels can impact the pain experience is a key concept for children to grasp. This may help them to understand that what they think and do can help them manage their pain [19, 20].

Use of relaxation techniques and empowering imagery may help a child to reduce his or her focus on pain [21]. Relaxing scenes should be tailored to the child's own likes, and where one child might focus on going to the circus as a relaxing experience, another might find this too stimulating. The counselor or health professional typically works with the child to select relaxing experiences and then assists the child in recalling all of the details of a relaxing scene, including specific sights, sounds, smells, and details about the sequence of events. Imagery, such as picturing a superhero who is battling pain symptoms, can serve to distract the child, which pain experts agree is a powerful pain management strategy [20–22]. Children may also use positive self-talk to overcome self-defeating statements, such as "I am always hurting because I have SLE." In contrast, the child can use positive self-statements, "I am feeling so happy and strong, and this makes me really strong to cope with my pain and feel better!" Teaching children that they can be problem solvers and use strategies to cope with their pain is a key message to boost children's self-efficacy for coping with pain [22, 23].

Relaxation strategies may help children cope with pain experiences [22]. Young children may have difficulty following complex relaxation strategies, but we have found that they are able to use a rock sponge activity, where they tense and relax their fist (provided this is not the area where pain symptoms are focused) to help them relax [21]. They also may tense and relax their legs or feet. We also work with children to talk about doing something fun, which may focus their thinking on something other than feelings of pain.

Some parents provide children with attention for pain behaviors, which accidentally reinforces pain complaints. Parents need to learn to reward their child for coping with his or her pain, adhering to doctors' recommendations, and following mild exercise regimes (being active can be a key pain management strategy). Involving children in physical exercise (as medically indicated) so that they become more active concomitantly reduces pain experiences [24]. Including strategies for parents, such as teaching parents to reinforce positive self-statements and ignore negative coping, should be emphasized. Research is lacking about whether age peers could help children with SLE by encouraging them to use pain management strategies. We believe that studies examining peers as supports in pain management efforts may be a fruitful area for research.

Degotardi and her colleagues evaluated a cognitive behavioral intervention for children with fibromyalgia [25]. The 8-week intervention consisted of education about the etiology of fibromyalgia; the role of stress and pain; how pain works in the body; and the relationship between emotional issues, sleep, and fatigue. Throughout the intervention (during multiple weeks), children learned to change negative thinking patterns and use positive thinking (such as reframing negative thoughts) to improve their functioning [25]. Children learned to set goals and were rewarded for changes in adaptive behaviors, in terms of managing their pain responses. Thus, goal setting, positive self-statements, changing negative thought patterns and self-talk, and use of distraction and relaxation were key strategies in the intervention package. Children in elementary school were included in the sample and it is noteworthy that interventions were tailored to key developmental issues for young children. Results were positive, indicating that children reported feeling more "in control" of their illness, felt less fatigue, and reported fewer disease-related symptoms. Parents reported that children's pain complaints lessened; children reported fewer somatic symptoms.

Walco and colleagues also used cognitive behavioral strategies to teach pain management strategies to 13 children with arthritis [23]. Pain management strategies included progressive muscle relaxation (tensing and relaxing muscle groups), meditative breathing, and guided imagery. Guided imagery occurs when an adult guides the child through imagining a relaxing scene, such as going to the beach or mountains. The guided imagery strategy in Walco et al.'s study served to distract children from thinking about pain [23]. Children can also generate positive images. An example might be a superhero, with superpowers to fight pain. This hero might have superpowers to "blow out" the hot fire of a pain experience. Parents who participated in the study were taught to reward coping behaviors and decrease attention paid to pain behaviors [23]. Future studies need to be conducted to determine if CBT strategies also are effective in reducing pain for children with SLE.

Brown et al. conducted a randomized controlled trial to deliver cognitive behavioral therapy to female adolescents with SLE [26]. Adolescents in this study were randomly assigned to receive health education, CBT, or be in a control group. The researchers had an impressive battery of assessment instruments to examine pain, psychosocial characteristics of participants, and quality of life. Those adolescents in the CBT group learned calming self-statements and techniques to diminish negative self-statements and affectivity associated with pain. Relaxation (progressive muscle relaxation, breathing), distraction (e.g., mental counting techniques), and problem-solving techniques were discussed with participants. Those in the education group learned about SLE and the importance of living a healthy lifestyle. Unfortunately, results of this study were not conclusive. Thus, reduced pain and improved quality of life were not discovered for the CBT group compared to the other two groups. However, the coping abilities of adolescents in the CBT group were significantly more advanced than those of females in the other two groups. The authors speculated that the dose of the intervention may not have been strong enough to produce intended results [26].

Our literature search also revealed studies using CBT interventions with adults. The interventions in these studies could also be used with children. Liang et al. conducted a systematic review of the literature investigating psychological interventions for adults with SLE [27]. They discovered six studies that were randomized controlled trials. After reviewing these studies, Liang et al. concluded that the psychological interventions were successful in reducing depression and improving the health status of participants with SLE. Two of the studies reviewed by Liang et al. presented information on CBT. These projects were conducted by Navarrete-Navarrete and colleagues and both presented valuable information on CBT [28, 29].

One study appeared particularly germane to the current paper. In this paper, Navarrete-Navarrete et al. discussed a CBT intervention for adults [28]. They randomly assigned 18 adults with SLE to a treatment group and 16 to a conventional care group. Those assigned to the treatment group participated in psychoeducational sessions. Participants learned CBT techniques including challenging and stopping negative thoughts, deep breathing for relaxation, and muscle relaxation. Participants in the treatment group reported higher quality of life after intervention and at a 15-month follow-up [28]. We believe the CBT techniques used for this project are easily transferable to children. For instance, children can be taught deep breathing through exercises with blowing bubbles. They can also learn to take slower breaths by slowly counting to three while inhaling, holding the breath (for a count of 3), and then slowly exhaling while counting to three. Children can learn to identify negative thoughts and replace negative thinking with positive statements. Finally, role-play or modeling can be used to teach children a thought-stopping exercise. Children can learn to identify a negative thought pattern, see a stop sign (to stop the negative thought), and then replace the negative thought with a positive image (e.g., going to a birthday party or an amusement park).

In another study with adults, Williams et al. developed an intervention to decrease stress in lupus patients [30]. Patients who were enrolled in the experimental group were involved in six weekly lessons drawn from the "Better Choice Better Health" Program developed by Stanford University [31, 32]. Meetings were facilitated by "nonhealth" professionals who

also had a chronic illness. Lessons covered nutrition; ideas for coping with pain, fatigue, and isolation; improving activity; and communicating effectively with others about illness. Results indicated improved health and reduced experiences of pain, stress, and depression. Feelings of self-efficacy for disease management improved, which can have a positive impact on adherence to the treatment regimen. The authors concluded that the intervention was successful but that further studies will be important to understand the impact of individual components of the intervention program. We believe that many aspects of this program, such as discussing healthy living, coping with pain and fatigue, and ideas for improving activity, could be used to educate children and their parents.

5. Conclusions

This paper has highlighted the importance of peer support and use of CBT interventions to manage pain as two potential interventions to enhance resilience for children with SLE. Medical professionals need to implement and study resilience in children with SLE to determine paths of success for these children as they cope with a serious chronic illness. Further research examining psychological interventions in combination with concurrent medication therapies in improving pain and quality of life is needed. Our literature review did not yield studies assessing whether peers can support children with SLE in pain management efforts. Future studies examining the influence of peer support and CBT strategies for pain management are needed. Studies examining the influence of each of the aforementioned variables will determine if these factors impact child quality of life, emotional functioning, and social development.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] R. Mina, M. S. Klein-Gitelman, A. Ravelli et al., "Inactive disease and remission in childhood-onset systemic lupus erythematosus," *Arthritis Care & Research*, vol. 64, no. 5, pp. 683–693, 2012.

[2] D. M. Levy and S. Kamphuis, "Systemic lupus erythematosus in children and adolescents," *Pediatric Clinics of North America*, vol. 59, no. 2, pp. 345–364, 2012.

[3] S. A. Kohut, J. Stinson, M. van Wyk, L. Giosa, and S. Luca, "Systematic review of peer support interventions for adolescents with chronic illness," *International Journal of Child and Adolescent Health*, vol. 7, no. 3, pp. 183–187, 2014.

[4] J. Reiter-Purtill, J. M. Waller, and R. Noll, "Empirical and theoretical perspectives on the peer relationships of children with chronic conditions," in *Handbook of Pediatric Psychology*, M. C. Roberts and R. C. Steele, Eds., pp. 672–688, Guilford Press, New York, NY, USA, 2009.

[5] D. J. McCarty, S. Manzi, T. A. Medsger Jr., R. Ramsey-Goldman, R. E. LaPorte, and C. K. Kwoh, "Incidence of systemic lupus erythematosus: race and gender differences," *Arthritis and Rheumatism*, vol. 38, no. 9, pp. 1260–1270, 1995.

[6] C. H. Feldman, L. T. Hiraki, J. Liu et al., "Epidemiology and sociodemographics of systemic lupus erythematosus and lupus nephritis among US adults with Medicaid coverage, 2000–2004," *Arthritis and Rheumatism*, vol. 65, no. 3, pp. 753–763, 2013.

[7] G. J. Pons-Estel, G. S. Alarcón, L. Scofield, L. Reinlib, and G. S. Cooper, "Understanding the epidemiology and progression of systemic lupus erythematosus," *Seminars in Arthritis and Rheumatism*, vol. 39, no. 4, pp. 257–268, 2010.

[8] J. Zhu, F. Wu, and X. Huang, "Age-related differences in the clinical characteristics of systemic lupus erythematosus in children," *Rheumatology International*, vol. 33, no. 1, pp. 111–115, 2013.

[9] A. Concannon, S. Rudge, J. Yan, and P. Reed, "The incidence, diagnostic clinical manifestations and severity of juvenile systemic lupus erythematosus in New Zealand Maori and Pacific Island children: the Starship experience (2000–2010)," *Lupus*, vol. 22, no. 11, pp. 1156–1161, 2013.

[10] K. H. Rubin and W. M. Bukowski, *Handbook of Peer Interactions, Relationships, and Groups*, Guilford Press, New York, NY, USA, 2011.

[11] C.-L. Dennis, "Peer support within a health care context: a concept analysis," *International Journal of Nursing Studies*, vol. 40, no. 3, pp. 321–332, 2003.

[12] M. Pinquart and D. Teubert, "Academic, physical, and social functioning of children and adolescents with chronic physical illness: a meta-analysis," *Journal of Pediatric Psychology*, vol. 37, no. 4, pp. 376–389, 2012.

[13] P. A. Forgeron, S. King, J. N. Stinson, P. J. McGrath, A. J. MacDonald, and C. T. Chambers, "Social functioning and peer relationships in children and adolescents with chronic pain: a systematic review," *Pain Research and Management*, vol. 15, no. 1, pp. 27–41, 2010.

[14] J. A. Smith and M. Osborn, "Pain as an assault on the self: an interpretative phenomenological analysis of the psychological impact of chronic benign low back pain," *Psychology and Health*, vol. 22, no. 5, pp. 517–534, 2007.

[15] H. Rhee, M. J. Belyea, J. F. Hunt, and J. Brasch, "Effects of a peer-led asthma self-management program for adolescents," *Archives of Pediatrics and Adolescent Medicine*, vol. 165, no. 6, pp. 513–519, 2011.

[16] M. McLaughlin, Y. Nam, J. Gould et al., "A videosharing social networking intervention for young adult cancer survivors," *Computers in Human Behavior*, vol. 28, no. 2, pp. 631–641, 2012.

[17] N. Al-Sheyab, R. Gallagher, J. Crisp, and S. Shah, "Peer-led education for adolescents with asthma in Jordan: a cluster-randomized controlled trial," *Pediatrics*, vol. 129, no. 1, pp. e106–e112, 2012.

[18] D. W. Jones, D. Wright, and T. A. Janowski, "What treatments relieve arthritis and fatigue associated with systemic lupus erythematosus?" *The Journal of Family Practice*, vol. 63, no. 10, pp. 607–617, 2014.

[19] J. Barlow, C. Wright, J. Sheasby, A. Turner, and J. Hainsworth, "Self-management approaches for people with chronic conditions: a review," *Patient Education and Counseling*, vol. 48, no. 2, pp. 177–187, 2002.

[20] P. A. McGrath, *Pain in Children: Nature, Assessment, and Treatment*, Guilford Press, New York, NY, USA, 1990.

[21] K. J. Rosenzweig and L. Nabors, "Pain coping strategies for children with arthritis," *BioMed Research International*, vol. 2013, Article ID 741428, 3 pages, 2013.

[22] M. Thastum, T. Herlin, and R. Zachariae, "Relationship of pain-coping strategies and pain-specific beliefs to pain experience in children with juvenile idiopathic arthritis," *Arthritis Care and Research*, vol. 53, no. 2, pp. 178–184, 2005.

[23] G. A. Walco, J. W. Varni, and N. T. Ilowite, "Cognitive-behavioral pain management in children with juvenile rheumatoid arthritis," *Pediatrics*, vol. 89, no. 6, pp. 1075–1079, 1992.

[24] A. C. E. de Blécourt, S. H. R. Preuper, C. P. van der Schans, J. W. Groothoff, and M. F. Reneman, "Preliminary evaluation of a multidisciplinary pain management program for children and adolescents with chronic musculoskeletal pain," *Disability and Rehabilitation*, vol. 30, no. 1, pp. 13–20, 2008.

[25] P. J. Degotardi, E. S. Klass, B. S. Rosenberg, D. G. Fox, K. A. Gallelli, and B. S. Gottlieb, "Development and evaluation of a cognitive-behavioral intervention for juvenile fibromyalgia," *Journal of Pediatric Psychology*, vol. 31, no. 7, pp. 714–723, 2006.

[26] R. T. Brown, S. R. Shaftman, B. C. Tilley et al., "The health education for lupus study: a randomized controlled cognitive-behavioral intervention targeting psychosocial adjustment and quality of life in adolescent females with systemic lupus erythematosus," *The American Journal of the Medical Sciences*, vol. 344, no. 4, pp. 274–282, 2012.

[27] H. Liang, X. Tian, L.-Y. Cao, Y.-Y. Chen, and C.-M. Wang, "Effect of psychological intervention on health-related quality of life in people with systemic lupus erythematosus: a systematic review," *International Journal of Nursing Sciences*, vol. 1, no. 3, pp. 298–305, 2014.

[28] N. Navarrete-Navarrete, M. I. Peralta-Ramírez, J. M. Sabio, I. Martínez-Egea, A. Santos-Ruiz, and J. Jiménez-Alonso, "Quality-of-life predictor factors in patients with SLE and their modification after cognitive behavioural therapy," *Lupus*, vol. 19, no. 14, pp. 1632–1639, 2010.

[29] N. Navarrete-Navarrete, M. I. Peralta-Ramírez, J. M. Sabio-Sánchez et al., "Efficacy of cognitive behavioural therapy for the treatment of chronic stress in patients with lupus erythematosus: a randomized controlled trial," *Psychotherapy and Psychosomatics*, vol. 79, no. 2, pp. 107–115, 2010.

[30] E. M. Williams, M. Penfield, D. Kamen, and J. C. Oates, "An intervention to reduce psychosocial and biological indicators of stress in African American lupus patients: the balancing lupus experiences with stress strategies study," *Open Journal of Preventive Medicine*, vol. 4, no. 1, pp. 22–31, 2014.

[31] K. Lorig, P. L. Ritter, and K. Plant, "A disease-specific self-help program compared with a generalized chronic disease self-help program for arthritis patients," *Arthritis & Rheumatism*, vol. 53, no. 6, pp. 950–957, 2005.

[32] K. R. Lorig, P. D. Mazonson, and H. R. Holman, "Evidence suggesting that health education for self-management in patients with chronic arthritis has sustained health benefits while reducing health care costs," *Arthritis & Rheumatism*, vol. 36, no. 4, pp. 439–446, 1993.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 435658, 10 pages
http://dx.doi.org/10.1155/2015/435658

Research Article

Prolactin and Dehydroepiandrosterone Levels in Women with Systemic Lupus Erythematosus: The Role of the Extrapituitary Prolactin Promoter Polymorphism at −1149G/T

Edward L. Treadwell,[1] Kenneth Wiley,[2] Beverly Word,[3] William Melchior,[3] William H. Tolleson,[3] Neera Gopee,[3] George Hammons,[3] and Beverly D. Lyn-Cook[3]

[1]*Brody School of Medicine, East Carolina University, Greenville, NC 27834, USA*
[2]*National Human Genome Research Institute, Rockville, MD 20892, USA*
[3]*FDA-National Center for Toxicological Research, Jefferson, AR 72079, USA*

Correspondence should be addressed to Beverly D. Lyn-Cook; beverly.lyn-cook@fda.hhs.gov

Received 2 January 2015; Revised 27 April 2015; Accepted 28 April 2015

Academic Editor: Lingyun Sun

Systemic lupus erythematosus (SLE) has shown an association with high levels of prolactin, low levels of dehydroepiandrosterone (DHEA), and induction of inflammatory cytokines in the serum of patients with the disease. This preliminary study examined the relevance of a −1149G/T functional single-nucleotide polymorphism (SNP) (rs1341239) in the promoter of the extrapituitary prolactin gene in a cohort of African American and European American women with lupus. Examination of this SNP revealed that the −1149TT genotype was correlated with higher levels of prolactin in serum and prolactin gene expression ($p = 0.0001$) in peripheral blood mononuclear cells (PBMCs). Lower levels of DHEA in serum were demonstrated in lupus patients ($p = 0.001$); those with the −1149TT genotype had the lowest levels of DHEA. Furthermore, a small subset of women who were on DHEA therapy and had a TT genotype showed a significant decrease in prolactin gene expression and lower disease activity scores (SLEDAI). Lupus patients, particularly African Americans, had significantly higher levels of IL-6 ($p = 0.0001$) and TNF-α ($p = 0.042$). This study suggests that the −1149TT genotype may be a risk factor for lupus and may predict who could possibly benefit from DHEA therapy; therefore, these results should be validated in a larger cohort with all ethnic groups.

1. Background

Systemic lupus erythematous (SLE) is a complex debilitating and fatal autoimmune disease affecting between 1.4 and 2.0 million Americans [1]. Hormonal, infectious, and environmental factors have been implicated in the etiology of the disease [2, 3]. Although the precise etiology of SLE remains elusive, the pathogenesis is often attributed to the development of antinuclear or anti-double stranded- (ds-) DNA autoantibodies [2, 4, 5]. Of the three types of lupus (systemic, discoid, and drug-induced), it is estimated that 70% of diagnosed lupus cases are systemic in which a major organ is affected in 50% of the cases, whereas discoid lupus accounts for approximately 10% of all other cases [1]. Genetic susceptibility to lupus has also been demonstrated by the fact that 20% of people with lupus will have a close relative (parent or sibling) who already has lupus or may develop lupus and, furthermore, approximately 5% of the children born to individuals with lupus will develop the illness [1]. Two noteworthy and perplexing aspects of SLE are its biases towards women and non-Europeans. SLE exhibits a female to male bias during prepubescence that increases from 4.5 : 1 in adolescence to 8–12 : 1 in adults and then declines to 2 : 1 in adults >60 years of age [2]. Women of childbearing age account for approximately 80–90% of SLE patients [6]. African American women experience 2–10-fold increased risk for developing SLE compared to European American women, develop symptoms at an earlier age, and have more severe symptoms and increased mortality [1, 2, 7, 8]. In fact, as

many as 1 in every 250 African American women is diagnosed with lupus [9].

Although few biomarkers have been discovered in early stages of lupus development, a number of biological changes have been noted in animal models or molecular epidemiological human studies [10]. High levels of prolactin have been associated with SLE and these levels have been correlated with a single-nucleotide polymorphism in the promoter of the prolactin gene [11]. However, a number of studies have shown conflicting results with this observation, likely due to different ethnic groups used in the various studies [12, 13].

Prolactin participates in a number of important functions in the body: it performs as a hormone, mainly due to its pituitary production, and it acts as a cytokine. It is prolactin's role as a cytokine, in which it participates in autocrine and paracrine actions that suggests an important role in the immune system. Prolactin is also secreted by immune cells and its receptor belongs to the family of cytokine receptors type 1 [14]. Although prolactin expression by the pituitary and other tissues utilizes the same gene, the promoters, regulatory regions, transcriptional control mechanisms, and final mRNA transcripts are tissue-dependent [15]. Studies have suggested its role in immunomodulation; however, the actual role of prolactin in the immune system remains unclear.

In addition to high levels of prolactin, lupus patients have low serum levels of dehydroepiandrosterone (DHEA). DHEA and its metabolite, dehydroepiandrosterone sulphate (DHEAS), are the major androgens secreted by the adrenal glands and are the precursor for estrogen and testosterone [16]. DHEA exerts antiproliferative and anti-inflammatory effects, and it modulates immune function [17]. In addition to low levels of DHEA in lupus patients, studies have shown low levels of DHEA in other inflammatory diseases [18].

One of the overall objectives of this study was to determine if the polymorphism in the promoter of the extrapituitary prolactin gene modulates expression levels of prolactin, particularly in African American women whose inclusion has been limited in other studies, and whether this polymorphism plays a role in patients' response to DHEA therapy. Levels of DHEA, DHEAS, estrogen, and testosterone in women with and without lupus were examined. Finally, this study also investigated whether ethnic differences were noted in IL-6 or TNF-α levels between European American women and African American women.

2. Methods

2.1. Human Blood Samples. Blood samples were obtained and processed as previously published from our laboratory [19] from a cohort of patients, after obtaining informed consent, who had been diagnosed with lupus according to the American College of Rheumatology criteria and were currently on routine therapy in addition to treatment with or without DHEA. This study consisted of a total of 256 patients, 87 African American females with lupus, 76 healthy age-matched controls (will be designated as nonlupus); 25 European American females with lupus, 33 healthy age-matched controls (will be designated as nonlupus); 10 African American males with lupus, 13 healthy age-matched controls (will be designated as nonlupus); and 5 European American males with lupus, 7 age-matched healthy controls (will be designated as nonlupus). These patients were a part of a larger LUPUS study at the Brody School of Medicine-East Carolina University, Greenville, NC. For consistency, all blood samples were obtained between 9:00 am and 12:00 pm, with a previous period of fasting and resting. Whole blood was collected in two portions: 10 mL whole blood was collected in plain or serum separator tubes for serum hormone and cytokine analyses and an additional 10 mL whole blood was collected in heparinized tubes for molecular biological studies. Blood and serum samples were shipped after collection to the National Center for Toxicological Research (NCTR) for molecular and clinical chemistry analyses. This study received IRB approval from East Carolina Brody School of Medicine and the FDA Research Involving Human Subject Committee (RIHSC).

2.2. Prolactin $T \rightarrow G^{-1149}$ Polymorphism. Genotyping was conducted using restriction fragment length polymorphism (RFLP) analyses and confirmed by DNA sequencing. Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using a modified QIAmp DNA Blood Maxi kit (QIAGEN, Valencia, CA). The polymorphism at position −1149 of the promoter of the extrapituitary PRL gene was amplified with the following primers: F-GAAGTTGAGCCTCAGGATGG and R-CTCAACAGCTTCTCAGTCAACA. PCR-RFLP analysis with *ApoI* digestion was conducted on the sequence for prolactin (GENBANK Accession number AF068856:GATAACCTGG AGAAAGGAGG AAAGATAATTTTATGGAGTT AGAGAGACA) that contained the prolactin polymorphism in the promoter region. All polymorphisms were confirmed with automatic sequencing.

2.3. RNA Isolation and Quantitative Real Time PCR. RNA was extracted as described previously [18] by using a PAXgene RNA kit (QIAGEN, Valencia, CA). After extraction, all RNA samples were tested for their integrity and concentration using a Bio-Rad Experion Automated Electrophoresis System (BIO-RAD, Hercules, CA). cDNAs were synthesized from total RNA extractions using a Clontech Advantage RT-for-PCR Kit (Clontech, Mountain View, CA). Prolactin expression analysis was conducted using a Bio-Rad IQ5 quantitative Real Time Polymerase Chain Reaction Detection System (BIO-RAD, Hercules, CA). GAPDH was used as an endogenous control. qRT-PCR conditions were as follows: 50°C for 2 minutes and 95°C for 10 minutes (95°C for 15 seconds, 62°C for 30 seconds, and 72°C for 30 seconds) for 47 cycles. Relative quantitation of prolactin and TNF-α mRNA expression was normalized to GADPH and fold changes were calculated using the $2^{-\Delta\Delta CT}$ method. Primers utilized for prolactin, TNF-α, and GAPDH are listed below:

Prolactin R: 5′ CGG CGC GGT CAA ACA GGT CT 3′.

Prolactin F: 5′ ACC AGG AAA AGG GAA ACG AAT GCC 3.

GAPDH-F: 5′ CCACCCATGGCAAATTCCATG 3′.

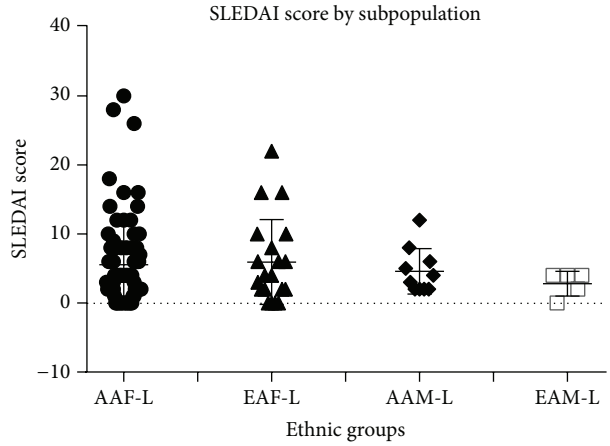

Figure 1: SLE disease activity index (SLEDAI) by population. African American (AAF-L) and European American (EAF-L) women with lupus have the highest SLEDAI scores compared to African American and European American men with lupus.

GAPDH-R: 5′ TCTAGACGGCAGGTCAGGTCC 3′TNF-alpha.

TNF-α-F: 5′-CTT CTC CTT CCT GAT CGT GG-3′.

TNF-α-R: 5′-GCT GGT TAT CTC TCA GCT CCA-3′.

2.4. Circulating Hormones and Cytokines. Testosterone, estradiol, prolactin, and DHEA-sulfate were assayed using Siemens RIA "Coat-A-Count" methods (Los Angeles, CA) and DHEA was assayed with Diagnostics Systems Laboratories (Webster, TX) using a coated tube RIA method. All tubes were counted on a PerkinElmer Cobra 5005 gamma counter (Shelton, CT). TNF alpha and IFN gamma were assayed using Antigenix America (Huntington Sta., NY) ELISA kits. All plates were read on the BioTex Instruments (Winooski, VT) Elx808 plate reader using Gen5 software for curve analysis and calculations.

2.5. Statistics. Differences in the frequencies of the GG, GT, and TT genotypes were analyzed using Fisher's exact test. For this study, a comparison analysis was conducted for each of the specific genes, serum levels, and cytokine profiles. The comparisons included patients with SLE compared to the control population and African Americans with SLE compared to European Americans with SLE. A two-tailed Mann-Whitney t-test was used to determine if significant differences existed. To compare values, p values of <0.05 were considered significant. All analyses were performed using GraphPad Prism (version 4) software (La Jolla, CA).

3. Results

Figure 1 shows the individual differences in the SLEDAI score by subpopulations in this study.

The highest disease activity within our study was found in African American and European American women with lupus. This study had a higher number of African American women compared to other published studies investigating the −1149G/T polymorphism. Higher levels of prolactin were noted in the serum of lupus patients ($p = 0.0026$) (Figure 2(a)). Prolactin gene expression levels were also higher in PBMCs from lupus patients ($p = 0.0017$) compared to nonlupus controls (Figure 2(b)).

Figure 3 shows a representative electrophoretic gel depicting RFLP analysis of the prolactin −1149 SNP.

Figure 4(a) shows that the genotype −1149TT was correlated with higher prolactin gene expression ($p = 0.0485$) in lupus (L) patients compared to age-matched nonlupus (NL) patients. An increase in prolactin levels was also noted in the serum prolactin protein ($p = 0.0230$) of these patients (Figure 4(b)). However, the −1149GT ($p = 0.1503$) and −1149GG ($p = 0.1480$) genotypes did not significantly correlate to any differences in prolactin gene expression levels in PBMCs from lupus patients when compared to age-matched nonlupus patients (Figures 4(c) and 4(d)).

In addition to high levels of prolactin, low levels of DHEA were detected in the serum of lupus patients. Patients with lupus had lower levels of DHEA when compared to age-matched controls ($p = 0.0001$) (Figure 5(a)). Lupus patients with the TT genotype had lower serum levels of DHEA than patients with the GG genotype ($p = 0.0367$) (Figure 5(b)). Furthermore, African American women with lupus with the TT genotype had significantly lower DHEA levels ($p = 0.0151$), when compared to female African American lupus patients with the GG or GT genotype (Figure 5(c)) or when compared to age-matched controls ($p = 0.022$) (Figure 5(d)).

When DHEA was given as therapy, patients had a lower disease activity index ($p = 0.0144$) (Figure 6(a)) and those with the TT genotype had an even lower disease activity index ($p = 0.0005$) compared to the GG genotype (Figure 6(b)). In addition, DHEA lowered the levels of prolactin in a selected group of patients (data not shown).

DHEA is thought to regulate proinflammatory cytokines; therefore, low DHEA levels could play a role in expression of high levels of cytokines, such as IL-6 and TNF-α. Our data showed (Figure 7(a)) higher levels of IL-6 expression in lupus patients ($p = 0.0001$) and that African American women with lupus had higher levels than European American women ($p = 0.0272$) (Figure 7(b)).

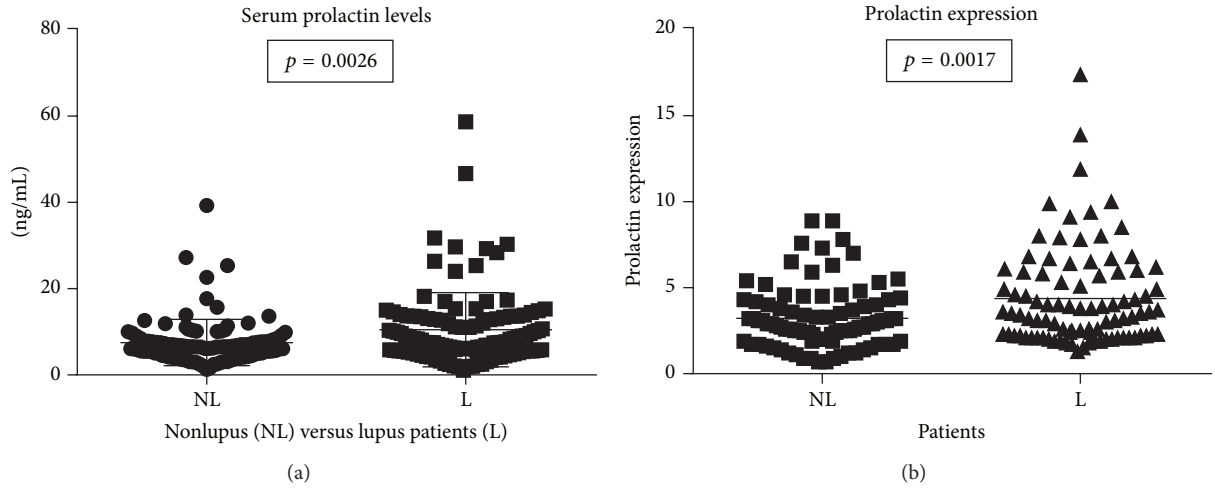

Figure 2: Prolactin expression and serum levels. Patients with lupus have significantly higher serum levels of prolactin ($p = 0.0026$) and expression at the mRNA level in PBMCs ($p = 0.0017$), although individual differences are shown among the patients.

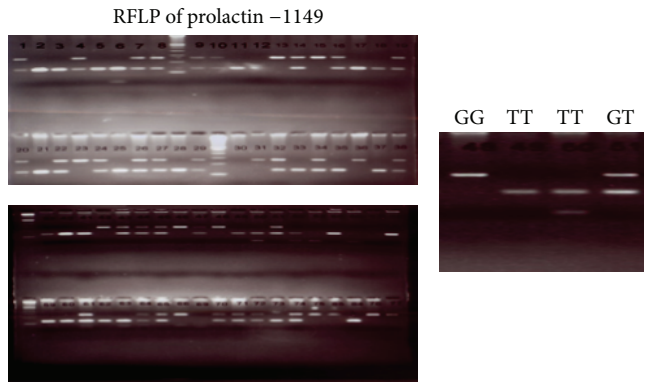

Figure 3: Restriction fragment length polymorphism (RFLP) of prolactin −1149. RFLP analysis with *ApoI* digestion yields three fragment lengths to distinguish the three genotypes, GG, GT, and TT.

African American women with lupus also had a higher level of TNF-α ($p = 0.0427$) (Figure 8).

In addition to high levels of prolactin and low levels of DHEA, results from this study revealed a significant increase in serum estradiol in lupus patients (Figure 9(a)) ($p = 0.0003$). There was a significant difference in estradiol levels in premenopausal (female young (FY)) (<50 years of age) and postmenopausal women (female old (FO)) (>50 years of age) ($p < 0.05$) when compared to their nonlupus counterpart at each menopausal status ($p < 0.05$), respectively (Figure 9(b)). Figure 9(c) further showed that lupus women with the TT genotype have a significant higher level of estradiol ($p = 0.0001$) when compared to the GG genotype. There was no difference in estradiol levels in lupus males compared to nonlupus males (data not shown).

No significant differences were found in testosterone levels between African American males and European American males with lupus ($p = 0.7639$) (Figure 10(a)); however, a small number of young women with lupus demonstrated higher levels of testosterone (Figure 10(b)).

4. Discussion

Prolactin's role in human autoimmune diseases remains a largely unexplored area in which research is greatly needed, particularly when investigating whether its involvement in different ethnic groups may cause different biological outcomes based on an individual's exposure and lifestyle factors. Prolactin's exact role in the physiology and pathogenesis of autoimmune diseases, such as lupus, has not been totally clarified. This protein acts as both a hormone and a cytokine depending on its biological context [14]. Prolactin is considered a cytokine due to its secretion by immune cells and by the fact that its receptors belong to a family of cytokine receptors type I.

Although prolactin is mainly expressed by the pituitary gland, extrapituitary promoter expression has been shown in a number of other organs [20]. Both pituitary and extrapituitary prolactin share the same gene but are under different promoters. Extrapituitary expression of prolactin is cell-specific and is independent of the Pit-1 transcription factor, which induces pituitary expression of prolactin

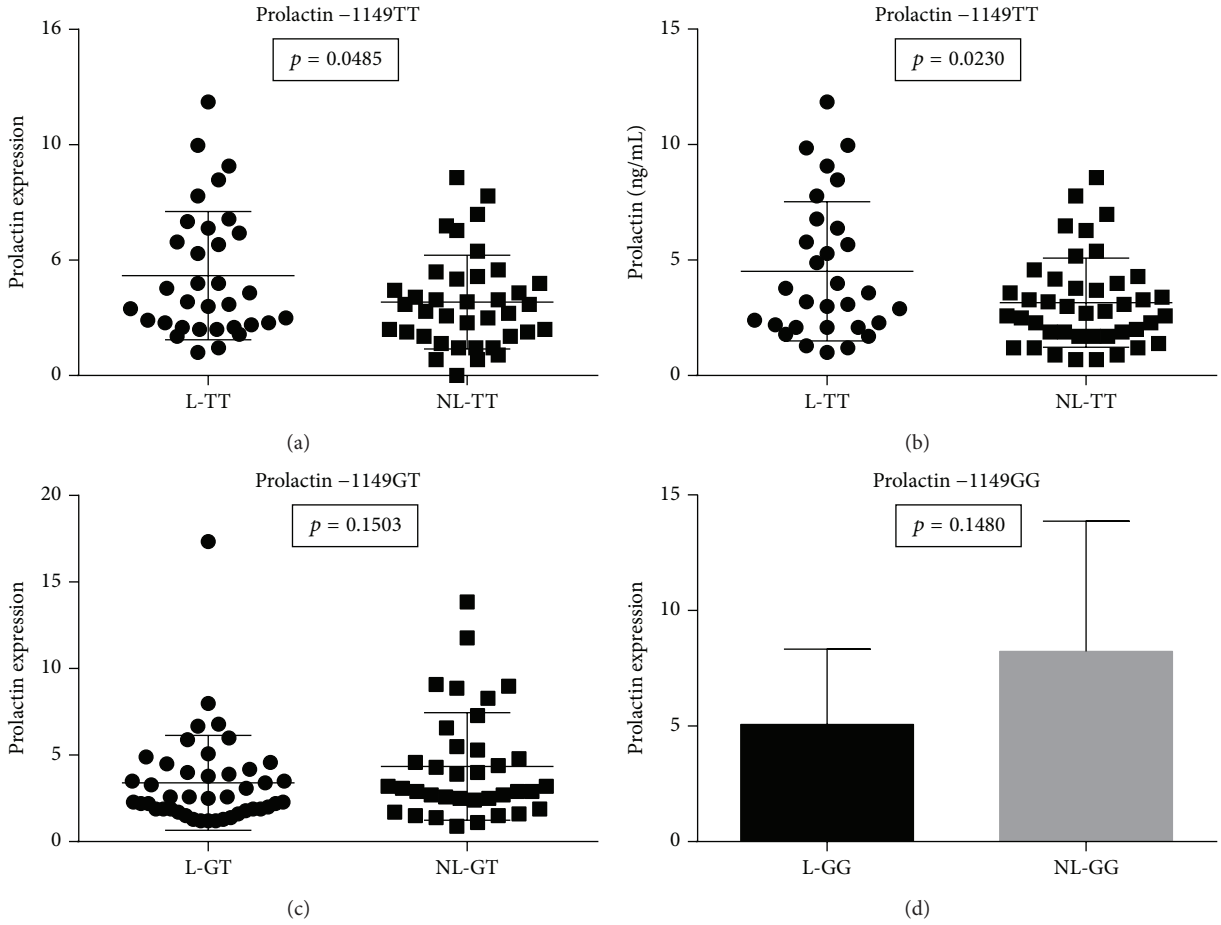

Figure 4: The genotype TT prolactin levels and expression. A significant number of lupus patients with the TT genotype demonstrated higher levels of prolactin expression (a) ($p = 0.0485$) and protein serum levels (b) ($p = 0.0230$). There were no significant differences in expression or serum levels in the other genotypes GT (c) and GG (d).

[21]. The extrapituitary promoter has been shown to contain a functional single-nucleotide polymorphism (SNP) at −1149G/T (rs1341239) in the GATA sequence. Studies have shown that the G allele leads to higher prolactin levels in lymphocytes in serum [22] and the GG genotype was associated with systemic lupus [23]; however, our study showed a different finding. The TT genotype was associated with higher expression of prolactin in lupus patients which may be due to the majority demographic in this study being African American women. Previous studies were done in different ethnic groups or had a very limited number of African American women while our study population was predominantly African American women.

This study further demonstrated that the lupus cohort in this study had a higher serum level of prolactin in their PBMCs in comparison to age-matched nonlupus patients. Not only was the TT genotype associated with higher expression of prolactin, but also these patients showed decreased levels of DHEA. DHEA and its metabolite dehydroepiandrosterone sulfate (DHEAS) are the most abundant circulating human adrenal steroids [16]. The critical role of low levels of DHEA in autoimmune diseases, such as lupus, rheumatoid arthritis, and other inflammatory diseases, remains an underexplored area of research; however, studies have indicated its involvement in improving overall immune function [24]. Animal studies have shown that high levels of proinflammatory cytokines, such as IL-6 and TNF-α, are restored to normal levels by DHEA administration [25]. It is believed that DHEA decreases TNFα and IL-6 through inhibition of NF-κB activation [26]. Higher levels of serum IL-6 were found in lupus patients in this study, and African American women had the highest level when compared to European American women and males. African American women also had the highest level of TNF-α when compared to nonlupus and lupus European American women and men in general. This study showed that, in a subgroup of women on DHEA therapy, those lupus patients with the TT genotype had lower levels of IL-6 expression compared to those patients on DHEA therapy with GG or GT genotypes. Furthermore, women with a TT genotype on DHEA therapy had a lower disease activity score. These results need further investigation with larger populations; however, they could explain the conflicting results shown in a number of studies or clinical trials using prasterone (DHEA) [27–29].

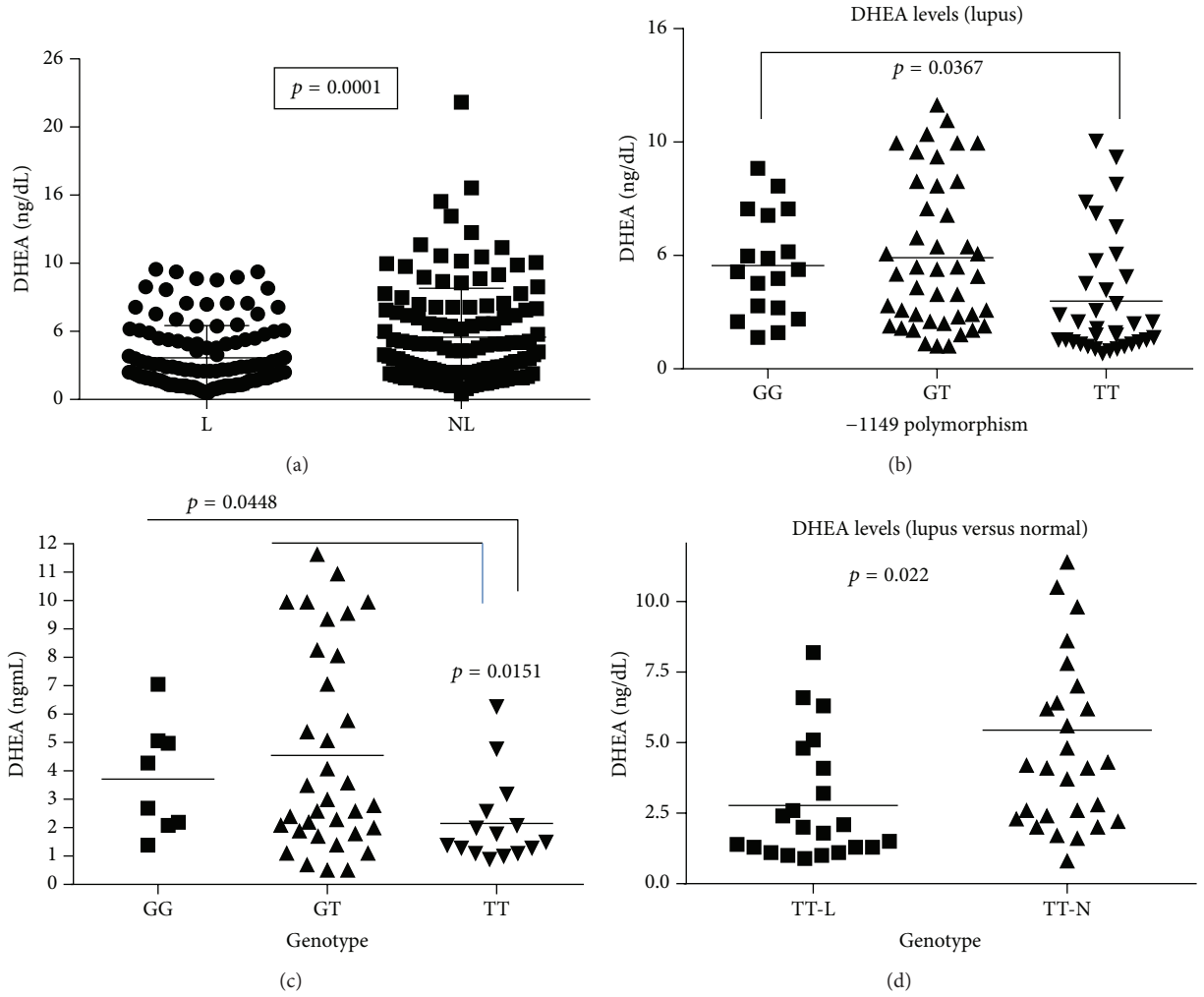

Figure 5: Serum levels of DHEA. (a) shows that lupus patients had a significant lower level of serum DHEA ($p = 0.0001$). (b) shows that the TT genotype had the lowest level of DHEA compared to GT or GG in lupus patients. Furthermore, (c) demonstrates that African American women with lupus and the TT genotype had the lowest level of DHEA. (d) shows that the serum DHEA level in TT genotype in African American women was significantly lower when compared to their normal age-matched nonlupus patients ($p = 0.022$).

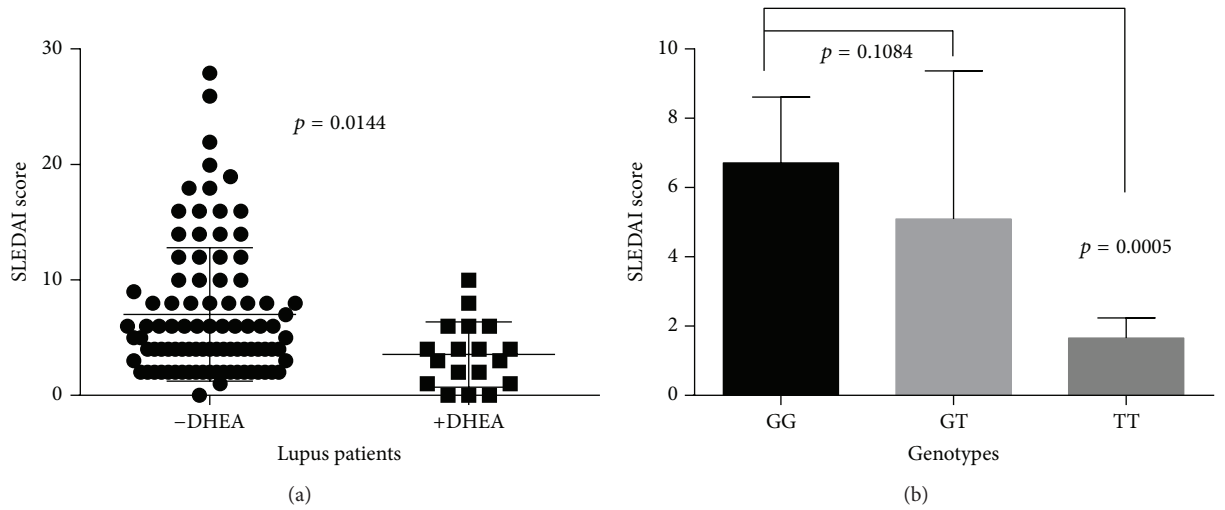

Figure 6: SLEDAI score and DHEA therapy. (a) shows that lupus patients on DHEA therapy had lower SLEDAI scores than patients not on therapy. (b) demonstrate that those lupus patients with a TT genotype and on DHEA therapy had lower disease activity scores compared to those with GT and GG genotypes.

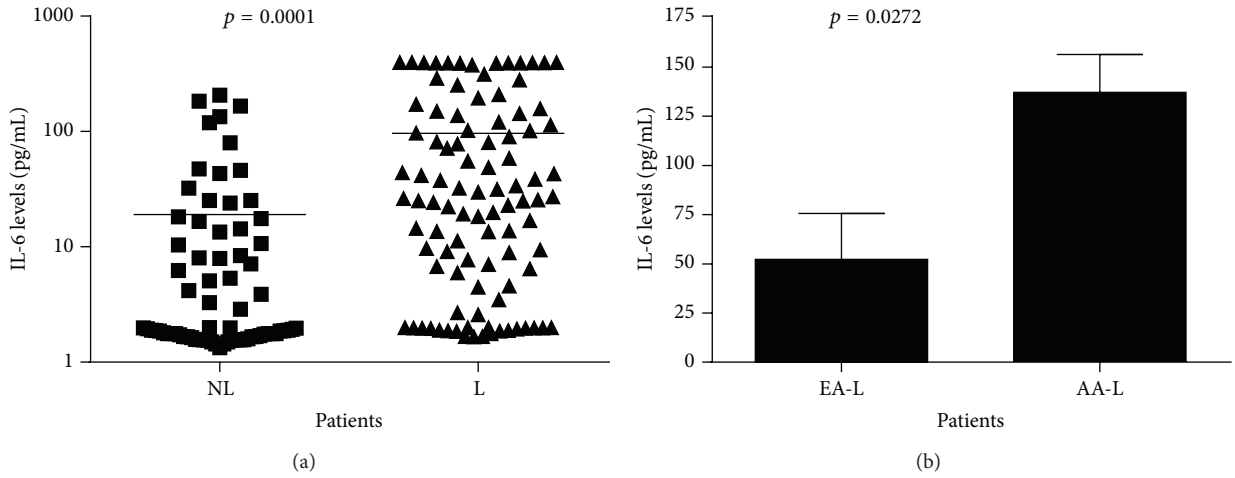

Figure 7: IL-6 serum levels in lupus and nonlupus patients. (a) shows that lupus patients have a significantly higher level of IL-6 compared to age-matched controls ($p = 0.0001$); however, (b) African American women with lupus have an increased level of IL-6 compared to European American women with lupus ($p = 0.0272$).

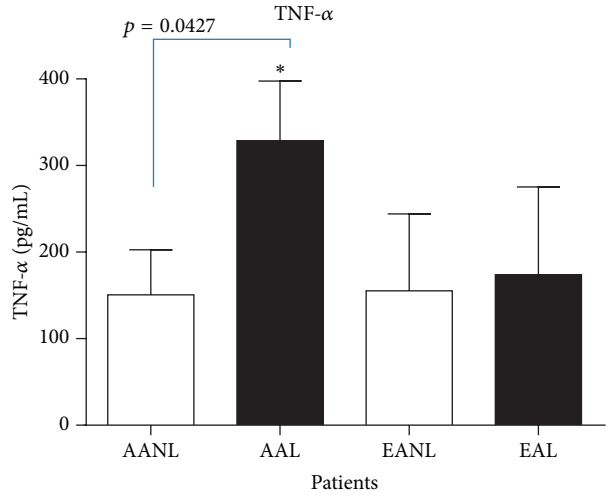

Figure 8: Tumor necrosis factor-alpha (TNF-α) serum levels. African American women with lupus (AAL) had the highest level of TNF-α compared to European American women with lupus ($p = 0.0427$).

This investigation has confirmed earlier reports of low levels of DHEA in lupus patients and high levels of prolactin. This study is the first to demonstrate a correlation between low levels of DHEA and high levels of prolactin and the TT genotype, particularly in African American women. A number of other studies that examined the extrapituitary polymorphism at −1149 showed that the GT was correlated with high levels of prolactin; however, those studies were done in populations other than African American women. Another finding in this study was that high levels of estradiol were also associated with the TT genotype and lupus patients in general had a higher level of estrogen when compared to their nonlupus counterparts, regardless of their menopausal status.

These findings in African Americans may give valuable insights into the lack of positive outcomes from various treatments and increased morality rates at younger ages. IL-6 and TNF-α expression levels were significantly higher in African American women. High levels of IL-6 have been associated with kidney damage in humans and in a number of animal models [30]. SLE is a chronic inflammatory disease that may affect any organ system in the body but it is the leading cause of kidney disease associated with death in young women [31]. IL-6 has both pro- and anti-inflammatory properties and increased IL-6 expression is a common response to tissue injury and organ failure [32]. Although this pathway has been targeted for novel therapeutic approaches, most drugs fail due to toxicity or other severe side effects.

These results warrant larger studies with diverse populations to fully understand the role of the functional single-nucleotide polymorphism −1149G/T in the promoter of the extrapituitary prolactin gene and its effects on autoimmunity. Also this study demonstrates that further studies should

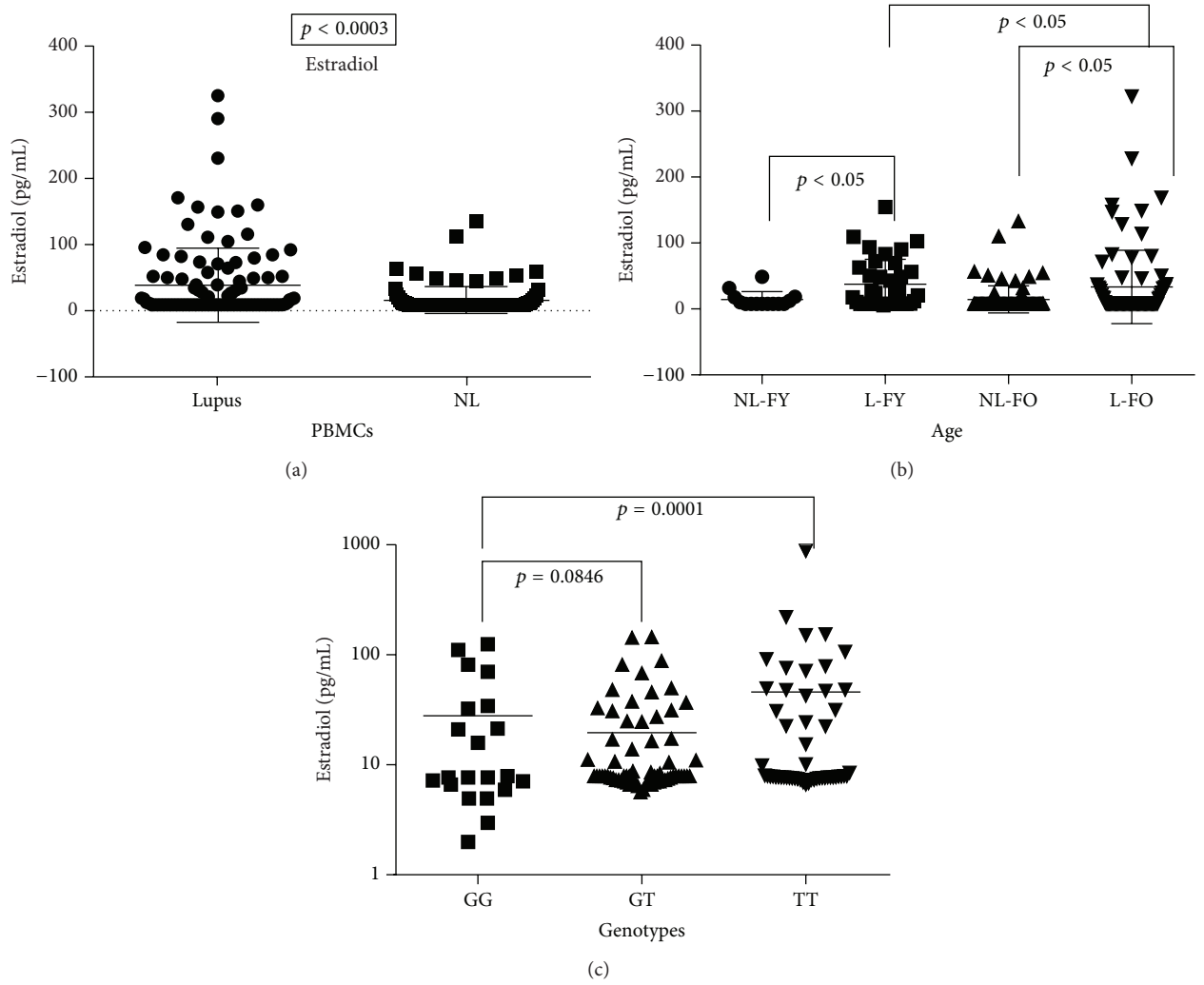

Figure 9: 17β-estradiol levels in serum. Estradiol levels in serum were significantly higher in lupus patients (a) ($p = 0.0003$). (b) However, women with lupus older (FO) than 50 had higher levels compared to women less than 50 (FY). (c) Lupus patients with the TT genotype had higher levels than women with the GG genotype.

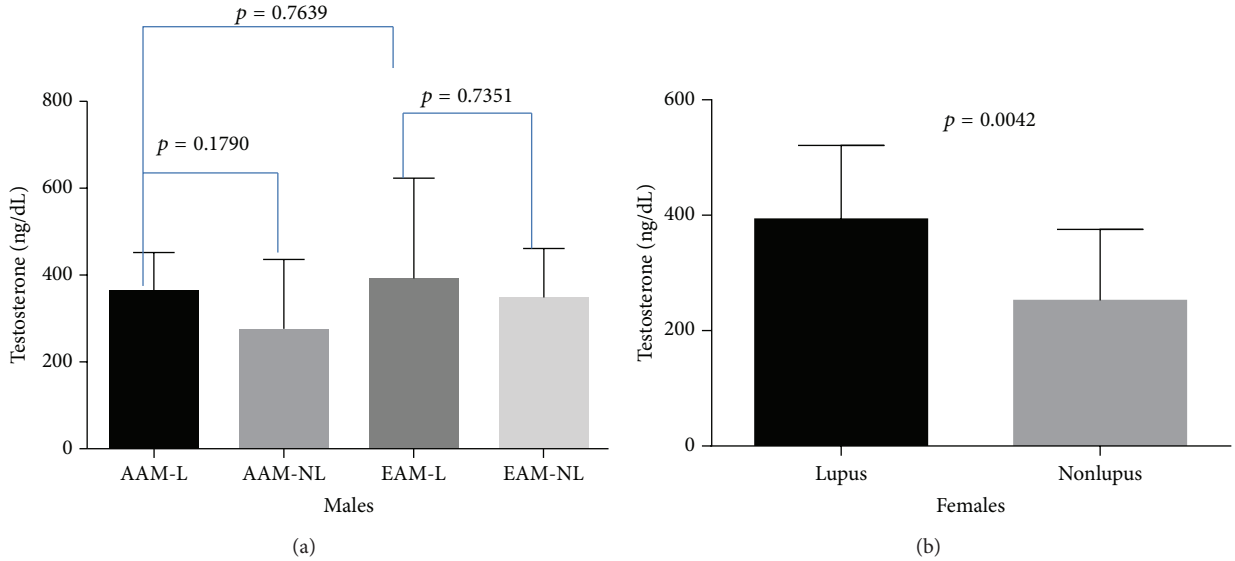

Figure 10: Testosterone levels in serum. (a) No difference was found in testosterone between the small numbers of males with lupus in this study; however, we did notice a higher level of testosterone in a small number of women less than fifty years of age.

be conducted on the mechanisms by which DHEA therapy is decreasing disease activity indexes in selected lupus patient with the TT genotype. Furthermore, more research is needed to determine if genotypes of prolactin extrapituitary promoter can be translated into clinical benefit, using a personalized medicine approach, for patients identification for DHEA therapy.

Disclaimer

The views presented in this paper do not necessarily reflect those of the US Food and Drug Administration.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] Lupus Foundation of America, *Statistics about Lupus*, Lupus Foundation of America, Washington, DC, USA, 2007, http://www.lupus.org/about/statistics-on-lupus.

[2] J. S. Cameron, "Lupus nephritis," *Journal of the American Society of Nephrology*, vol. 10, no. 2, pp. 413–424, 1999.

[3] C. C. Mok and C. S. Lau, "Pathogenesis of systemic lupus erythematosus," *Journal of Clinical Pathology*, vol. 56, no. 7, pp. 481–490, 2003.

[4] S. K. Datta, L. Zhang, and L. Xu, "T-helper cell intrinsic defects in lupus that break peripheral tolerance to nuclear autoantigens," *Journal of Molecular Medicine*, vol. 83, no. 4, pp. 267–278, 2005.

[5] J. S. Smolen, "Therapy of systemic lupus erythematosus: a look into the future," *Arthritis Research & Therapy*, vol. 4, supplement 3, pp. S25–S30, 2002.

[6] R. G. Lahita, "Gender and age in lupus," in *Systemic Lupus Erythematosus*, R. G. Lahita, Ed., Academic Press, San Diego, Calif, USA, 2004.

[7] T. Patavino and D. M. Brady, "Natural medicine and nutritional therapy as an alternative treatment in systemic lupus erythematosus," *Alternative Medicine Review*, vol. 6, no. 5, pp. 460–471, 2001.

[8] S. L. E. Lupus Foundation, *Facts about Gender and Racial Disparities in Lupus: Lupus Targets Women and Minorities*, 2007, http://www.lupusny.org/who.php.

[9] National Institutes of Health and National Institute of Arthritis and Muscoloskeletal and Skin Diseases, *Genetic Differences Found in African American, European American Lupus Families*, National Institutes of Health, 2002, http://www.niams.nih.gov/ne/highlights/spotlight/2002/lupusgene.htm.

[10] S. P. Crampton, P. A. Morawski, and S. Bolland, "Linking susceptibility genes and pathogenesis mechanisms using mouse models of systemic lupus erythematosus," *Disease Models & Mechanisms*, vol. 7, no. 9, pp. 1033–1046, 2014.

[11] M. Fojtíková, P. Čejková, R. Bečvář, J. Vencovský, J. Tomasová Studýnková, and M. Černá, "Polymorphism of the extrapituitary prolactin promoter and systemic sclerosis," *Rheumatology International*, vol. 30, no. 12, pp. 1691–1693, 2010.

[12] O. Vera-Lastra, L. J. Jara, and L. R. Espinoza, "Prolactin and autoimmunity," *Autoimmunity Reviews*, vol. 1, no. 6, pp. 360–364, 2002.

[13] D. Buskila, M. Lorber, L. Neumann, D. Flusser, and Y. Shoenfeld, "No correlation between prolactin levels and clinical activity in patients with systemic lupus erythematosus," *Journal of Rheumatology*, vol. 23, no. 4, pp. 629–632, 1996.

[14] E. Peeva, D. Michael, J. Cleary, J. Rice, X. Chen, and B. Diamond, "Prolactin modulates the naive B cell repertoire," *Journal of Clinical Investigation*, vol. 111, no. 2, pp. 275–283, 2003.

[15] B. Gellersen, R. Kempf, R. Telgmann, and G. E. DiMattia, "Non-pituitary human prolactin gene transcription is independent of Pit-1 and differentially controlled in lymphocytes and in endometrial stroma," *Molecular Endocrinology*, vol. 8, no. 3, pp. 356–373, 1994.

[16] J. S. Dillon, "Dehydroepiandrosterone, dehydroepiandrosterone sulfate and related steroids: their role in inflammatory, allergic and immunological disorders," *Current Drug Targets: Inflammation and Allergy*, vol. 4, no. 3, pp. 377–385, 2005.

[17] C. C. G. Chen and C. R. Parker Jr., "Adrenal androgens and the immune system," *Seminars in Reproductive Medicine*, vol. 22, no. 4, pp. 369–377, 2004.

[18] M. Charlton, P. Angulo, N. Chalasani et al., "Low circulating levels of dehydroepiandrosterone in histologically advanced nonalcoholic fatty liver disease," *Hepatology*, vol. 47, no. 2, pp. 484–492, 2008.

[19] K. L. Wiley, E. Treadwell, K. Manigaba, B. Word, and B. D. Lyn-Cook, "Ethnic differences in DNA methyltransferases expression in patients with systemic lupus erythematosus," *Journal of Clinical Immunology*, vol. 33, no. 2, pp. 342–348, 2013.

[20] C. Bole-Feysot, V. Goffin, M. Edery, N. Binart, and P. A. Kelly, "Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice," *Endocrine Reviews*, vol. 19, no. 3, pp. 225–268, 1998.

[21] V. Goffin, N. Binart, P. Touraine, and P. A. Kelly, "Prolactin: the new biology of an old hormone," *Annual Review of Physiology*, vol. 64, pp. 47–67, 2002.

[22] M. Fojtíková, M. Černá, P. Čejková, Š. Růžičková, and C. Dostál, "Extrapituitary prolactin promoter polymorphism in Czech patients with systemic lupus erythematosus and rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 66, no. 5, pp. 706–707, 2007.

[23] A. Stevens, D. Ray, A. Alansari et al., "Characterization of a prolactin gene polymorphism and its associations with systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 44, no. 10, pp. 2358–2366, 2001.

[24] A. H. Sawalha and S. Kovats, "Dehydroepiandrosterone in systemic lupus erythematosus," *Current Rheumatology Reports*, vol. 10, no. 4, pp. 286–291, 2008.

[25] S. J. Mills, J. J. Ashworth, S. C. Gilliver, M. J. Hardman, and G. S. Ashcroft, "The sex steroid precursor DHEA accelerates cutaneous wound healing via the estrogen receptors," *Journal of Investigative Dermatology*, vol. 125, no. 5, pp. 1053–1062, 2005.

[26] R. H. Straub, L. Konecna, S. Hrach et al., "Serum dehydroepiandrosterone (DHEA) and DHEA sulfate are negatively correlated with serum interleukin-6 (IL-6), and DHEA inhibits IL-6 secretion from mononuclear cells in man in vitro: possible link between endocrinosenescence and immunosenescence," *The Journal of Clinical Endocrinology & Metabolism*, vol. 83, no. 6, pp. 2012–2017, 1998.

[27] W. Marder, E. C. Somers, M. J. Kaplan, M. R. Anderson, E. E. Lewis, and W. J. McCune, "Effects of Prasterone (dehydroepiandrosterone) on markers of cardiovascular risk and bone turnover in premenopausal women with systemic lupus

erythematosus: a pilot study," *Lupus*, vol. 19, no. 10, pp. 1229–1236, 2010.

[28] R. F. van Vollenhoven, E. G. Engleman, and J. L. Mcguire, "An open study of dehydroepiandrosterone in systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 37, no. 9, pp. 1305–1310, 1994.

[29] R. F. van Vollenhoven, L. M. Morabito, E. G. Engleman, and J. L. McGuire, "Treatment of systemic lupus erythematosus with dehydroepiandrosterone: 50 patients treated up to 12 months," *Journal of Rheumatology*, vol. 25, no. 2, pp. 285–289, 1998.

[30] E. M. Simmons, J. Himmelfarb, M. T. Sezer et al., "Plasma cytokine levels predict mortality in patients with acute renal failure," *Kidney International*, vol. 65, no. 4, pp. 1357–1365, 2004.

[31] Y. Nechemia-Arbely, D. Barkan, G. Pizov et al., "IL-6/IL-6R axis plays a critical role in acute kidney injury," *Journal of the American Society of Nephrology*, vol. 19, no. 6, pp. 1106–1115, 2008.

[32] S. Rose-John, G. H. Waetzig, J. Cheller, J. Grötzinger, and D. Seegert, "The IL-6/sIL-6R complex as a novel target for therapeutic approaches," *Expert Opinion on Therapeutic Targets*, vol. 11, no. 5, pp. 613–624, 2007.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 742402, 8 pages
http://dx.doi.org/10.1155/2015/742402

Research Article

Automated Evaluation of *Crithidia luciliae* Based Indirect Immunofluorescence Tests: A Novel Application of the EUROPattern-Suite Technology

Stefan Gerlach, Kai Affeldt, Lena Pototzki, Christopher Krause, Jörn Voigt, Johanna Fraune, and Kai Fechner

Institute for Experimental Immunology, EUROIMMUN, Seekamp 31, 23560 Lübeck, Germany

Correspondence should be addressed to Kai Fechner; k.fechner@euroimmun.de

Received 3 April 2015; Accepted 14 June 2015

Academic Editor: Xuan Zhang

Systemic lupus erythematosus (SLE) is a severe rheumatic autoimmune disease with various clinical manifestations. Anti-dsDNA antibodies are an important immunological hallmark of SLE and their occurrence represents a major criterion for the diagnosis. Among the commonly applied test systems for determination of anti-dsDNA antibodies, the indirect immunofluorescence test (IIFT) using the flagellated *kinetoplastida Crithidia luciliae* is considered to be highly disease specific at moderate sensitivity. Since IIFT, however, is claimed to be affected by subjective interpretation and a lack of standardization, there has been an increasing demand for automated pattern interpretation of immunofluorescence reactions in recent years. Corresponding platforms are already available for evaluation of anti-nuclear antibody (ANA) IIFT on HEp-2 cells, the recommended "gold standard" for ANA screening in the diagnosis of various systemic rheumatic autoimmune diseases. For one of these systems, the "EUROPattern-Suite" computer-aided immunofluorescence microscopy (CAIFM), automated interpretation of microscopic fluorescence patterns was extended to the *Crithidia luciliae* based anti-dsDNA IIFT.

1. Introduction

For diagnosis of systemic lupus erythematosus (SLE), determination of autoantibodies is of significant diagnostic importance [1, 2]. Among these, antibodies against double-stranded DNA (anti-dsDNA) play a major role. Their presence constitutes an important immunological criterion for the diagnosis of SLE as stated by the American College of Rheumatology in 1982 [3]. A more recent approach by the Systemic Lupus Collaborating Clinics to revise and validate the American College of Rheumatology SLE classification criteria approved anti-dsDNA as a major serological feature of SLE, considering them as very specific and a marker of disease activity and kidney involvement [4]. Accordingly, studies in mice and humans provided evidences for a role of anti-dsDNA in the pathogenesis of lupus nephritis [2, 5–9].

Information on the prevalence of anti-dsDNA in SLE varies between studies, ranging from 30% to 98% [2, 10]. The application of different laboratory tests is one cause which contributes to this deviation [11, 12]. The most common methods for the detection of anti-dsDNA are enzyme-linked immunosorbent assays (ELISA), radio immunoassays (RIA, e.g., Farr assays and PEG assays) and *Crithidia luciliae* indirect immunofluorescence tests (CLIFT) [13]. It is hypothesized that each of these detects individual, yet overlapping, subgroups of anti-dsDNA revealing divergent properties (e.g., avidity, structural specificity) and, of particular interest, different clinical associations [10, 14]. Classical anti-dsDNA ELISA is accepted as the most sensitive but often less specific method for SLE diagnostics. Through modifications of the applied DNA substrates and their linkage to the test wells, an increase in diagnostic accuracy of the ELISA for SLE could be achieved in recent years [15, 16]. Nevertheless, consistency between different ELISA kits seems to be limited [12]. Therefore, primary test results usually require confirmation by a second assay such as Farr immunoassay and/or CLIFT, both of which are regarded as highly disease specific, detecting only antibody subpopulations with a high positive predictive

value for SLE [10, 12, 14, 17–19]. Since RIA employ radioactive elements, CLIFT is commonly considered as more applicable confirmatory test system in the clinical routine of SLE diagnostics [20].

CLIFT utilizes the protist *Crithidia luciliae* as substrate, taking advantage of its kinetoplast, a network of tightly packed dsDNA within a large mitochondrion. In contrast to the nucleus, the kinetoplast contains fewer proteins and thus allows a more selective detection of anti-dsDNA antibodies [21]. Sensitivities of the assay have been reported to range from around 30% to nearly 60% at very high disease specificities of typically above 95% [12, 14–16]. Therefore, CLIFT is appreciated as a useful tool to support the diagnosis of SLE and its discrimination from other diseases.

A limitation of CLIFT however is—as generally applies to the procedure of indirect immunofluorescence tests (IIFT)—the manual read-out of fluorescence signals and its subjective interpretation which lead to a high intra- and interlaboratory variability [12, 22–25]. Great efforts, therefore, have been made in previous years to develop automated solutions enabling optimal image acquisition as well as objective and standardized evaluation of immunofluorescence results, especially in the major field of ANA diagnostics [24, 26, 27]. IIFT on HEp-2 cells still is the recommended "gold standard" for ANA determination [28–30]. Thus, several commercial platforms for automated immunofluorescence microscopy have been developed and validated [31–37]. The automation was shown to greatly contribute to standardization and facilitation of ANA HEp-2 IIFT interpretation. Particularly with regard to positive/negative discrimination, the new systems achieved a very high consensus with manual result interpretation [38–41].

Among these platforms, the EUROPattern-Suite (Euroimmun AG, Lübeck, Germany) is a system for computer-aided immunofluorescence microscopy, combining several hardware and software modules for fully automated image acquisition and evaluation. It performs reliable discrimination of positive and negative ANA HEp-2 (and HEp-20-10) IIFT results. Additionally it provides the option of automated and accurate recognition of several single as well as mixed ANA patterns and titer estimation [32, 39, 40, 42]. Results and corresponding images are displayed within a user-friendly graphical interface (GUI) which allows interactive revision and requires final validation by the professional operator. Thus, the system can reach full compliance with visual immunofluorescence microscopy in terms of result interpretation. In comparison to classical microscopy, the EUROPattern-Suite requires less hands-on effort and is much more resistant to human error. A detailed description of the technology and its associated laboratory management system EUROLabOffice is provided by Krause et al. [42].

Here, we present the first data on automated fluorescence interpretation of CLIFT using the EUROPattern-Suite. Only very few systems have been described in this context in the literature so far [34, 43, 44].

2. Material and Methods

2.1. Human Sera. A panel of 569 consecutive human sera which were sent to an immunological reference laboratory (Lübeck, Germany) for routine anti-dsDNA screening as well as 100 sera of healthy blood donors were examined. Samples were blinded for the analysis, which was carried out in accordance to the ethical guideline stated in the Declaration of Helsinki (1964).

2.2. Crithidia luciliae Indirect Immunofluorescence Test (CLIFT). Indirect immunofluorescence on *Crithidia luciliae* was performed using the Crithidia luciliae (anti-dsDNA) EUROPattern kit following the manufacturer's instruction (Euroimmun AG, Lübeck, Germany). One slide contains 10 reaction areas, each provided with one biochip (2×2 mm fragments of coated cover slip glued into the reaction fields), coated with cells of the protist. Slides were manually incubated and washed with the help of the TITERPLANE technique. Samples were applied at a dilution of 1 : 10 in PBS-Tween. Fluorescein isothiocyanate (FITC-) labeled goat anti-human IgG was used for green fluorescent staining. Antiserum was supplied with Evans blue, used for red fluorescent counterstaining of the cells.

2.3. Evaluation of Anti-dsDNA Antibodies. A focused image of each biochip on the incubated slides was automatically taken by the EUROPattern fluorescence microscope (see description below). Images were then interpreted in terms of sample positivity/negativity, once automatically by the EUROPattern software and, in a parallel approach, visually by two experts working independently of each other and without notice of software results. Disagreements between visual results were decided by a third opinion. Anti-dsDNA titers of $\geq$1 : 10 were considered positive.

2.4. Description of the System. A detailed description of the general EUROPattern-Suite hardware and software composition is provided in [32, 42].

A new classification software has been specifically developed for the recognition and interpretation of anti-dsDNA on *Crithidia luciliae*: Two images per biochip, one in the green and one in the red fluorescence channel of the microscope, are taken at a 400-fold magnification, using the 40x microscope objective. On average, this magnification leads to the recording of 30 cells per image. Autofocusing is performed using transmitted light to avoid fluorescence bleaching. The underlying algorithm for subsequent fine adjustments has been adapted to the needs of *Crithidia luciliae* image acquisition, resulting in a focused fluorescence image at a resolution of 2,448 × 2,048 pixels within 18 seconds. Thus, a slide containing 10 biochips is processed in less than three minutes. Since the EUROPattern microscope is equipped with two cameras, corresponding images in the green (specific FITC fluorescence signal) and the red (Evans blue counterstaining) fluorescence channel are taken at the same time.

The following image classification process operates asynchronously, meaning that the software already provides the first results for interactive verification while the microscope is still running. The procedure incorporates multiple steps which are performed in sequential order (Figure 1).

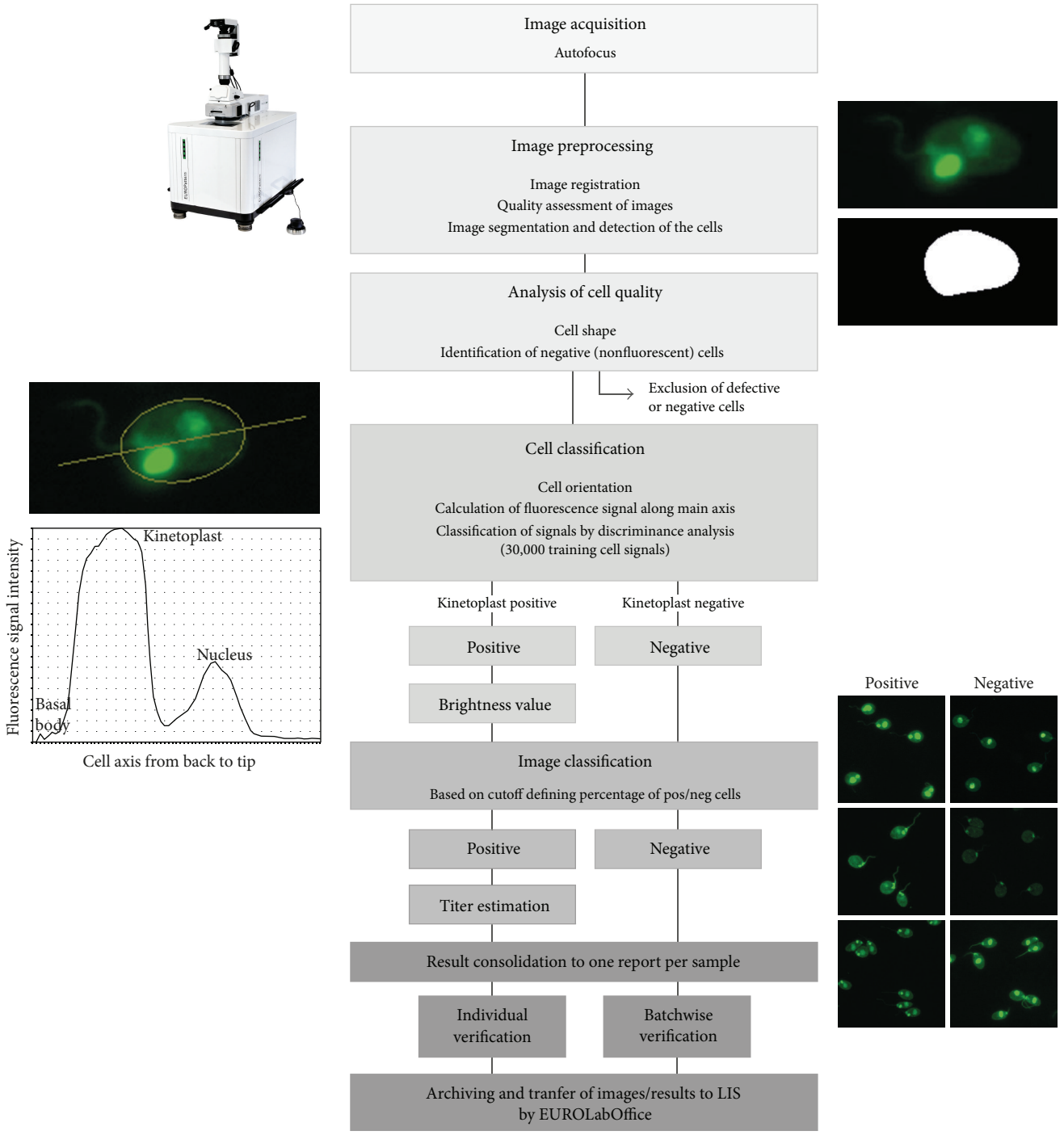

Figure 1: Flowchart of EUROPattern-Suite algorithm for computer-aided immunofluorescence microscopy of *Crithidia luciliae* IFT applied for the detection of antibodies against dsDNA.

(i) *Image Preprocessing*. Images of the green and red channel are loaded to memory and a perfect overlay of each image pair is generated which is important for subsequent image segmentation. Images are then analyzed regarding focal imprecision and potential incubation artefacts by application of specific software algorithms incorporating convolutional filter. Controlling the image sharpness is performed in the red fluorescence channel in which every cell can be detected. This strategy allows reliable discrimination between images of anti-dsDNA negative cells and images taken out of focus. As assessed by an internal visual validation, less than 0.1% of acquired images may reveal some degree of focal inaccuracy. Afterwards, image segmentation and precise detection of any cell are again performed in the red fluorescence

channel. Adaptive thresholding techniques are used to mask as well as select every cell by means of connected components.

(ii) *Analyzing Cell Quality*. Each cell mask is now examined in terms of shape characteristics and potential defects, such as size, ellipse form, aspect ratio, or defects of the convex hull. Defective cells are excluded from subsequent evaluation. Additionally, negative cells are identified by application of a threshold-based algorithm which assesses the brightness of the complete cell. If all cells are "dark," the complete image is set to negative and not processed any further.

(iii) *Cell Classification*. For this purpose, the kinetoplasts of the recognized and fluorescent cells need to be identified. This is achieved by the following steps. First, the software determines the orientation for each cell. Afterwards, a discrete and normalized signal is generated along the calculated main axis, based on mathematical measures such as mean value and standard deviation. Due to the set order of the cellular organelles (basal body, kinetoplast, and nucleus), the signal encodes their specific fluorescence intensities and even discloses the case of an organelle being absent. In a second step, extracted normalized signals are now classified by means of a discriminance analysis based on a reference training database. This database contains images of 30,000 incubated cells which have been acquired from incubations in the reference laboratory or in the context of validation studies. Each of the training images has been labelled by experts with specific information indicating the presence or absence of any fluorescent cell organelle. As a result of this classification, every cell is assigned as either positive or negative according to the fluorescence status of the kinetoplast. Furthermore, a brightness value is extracted from the kinetoplast fluorescence signal.

(iv) *Image Classification*. Images are classified into anti-dsDNA positive or negative, based on a configurable cutoff which defines the required percentage of positive cells within the image. Titer estimation per image is achieved by aggregating the cell brightness values and transforming them into an antibody titer (given in configurable titer steps). A confidence value is calculated which corresponds to the probability of the proposed results. Classifications and titer proposals concerning different dilutions of the same sample are merged into one final result which is given adjacent to the corresponding images within the GUI at the computer screen. The software generated result has to be verified by the physician and confirmed by one mouse click. This verification can be performed batchwise for negative samples and is executed one by one for positive samples.

3. Results

Beneficial automation of IIFT evaluation in a diagnostic laboratory requires reliable interpretation of the fluorescent images in clinical routine, encompassing high accuracy of software derived results compared to classical visual inspection by an expert and efficient operation and processing of the system. In case of CLIFT, this means accurate discrimination between positive and negative anti-dsDNA findings according to the presence or absence of a fluorescent *Crithidia luciliae* kinetoplast (Figure 2) within a time- and labor-saving evaluation work flow.

Table 1: Comparison of software-generated and visual positive/negative classification including 669 analyzed samples.

$n = 669$		Visual evaluation		Σ
		Positive	Negative	
EUROPattern	Positive	**73**	*19*	92
	Negative	*0*	**577**	577
	Σ	73	596	669
Sensitivity	100%			
Specificity	96.8%			
Accuracy	97.2%			

Concordant results are presented in bold font, differing results in italic font.

Therefore, the performance of the EUROPattern-Suite (CLIFT classification software) has been validated on the basis of a large number of consecutive sera which have been tested for anti-dsDNA with the help of a commercial CLIFT kit (Euroimmun AG, Lübeck, Germany). Images of all samples were automatically taken by the EUROPattern microscope. Every image revealed a high focal precision, as determined by the software algorithm (see Section 2), thus all of them were suited for subsequent evaluation.

3.1. Positive/Negative Classification (All Samples). Images of 669 tested samples in total were evaluated in terms of positivity/negativity either automatically by EUROPattern software or visually by two experts for CLIFT interpretation (Table 1). Visual inspection yielded 73 anti-dsDNA positive and 596 anti-dsDNA negative sera. Software generated results were 100% accurate with respect to positivity implying that the system likewise recognized the same 73 anti-dsDNA positive samples. Of note, 93% of the EUROPattern titer proposals for positive samples were concordant to visual estimations within the scope of reproducibility of immunofluorescence assays (+/−1 titer level, data not shown). Out of the 596 anti-dsDNA negative samples, the software correctly recognized 577 cases. The remaining 19 samples were determined to be negative by eye but positive by the software. Overall results correlate to a 100% sensitive and 96.8% specific determination of anti-dsDNA on *Crithidia luciliae* by the EUROPattern-Suite. Compared to visual microscopy, overall accuracy of the software was as high as 97.2%.

4. Discussion

The need for standardization and automation of IIFT is tremendous in all fields of autoimmune diagnostics in order to ensure objective antibody determination. The technological progress has generated automatic solutions for incubation and processing of slides and, primarily concerning ANA diagnostics on HEp-2 cells, even imaging and evaluation

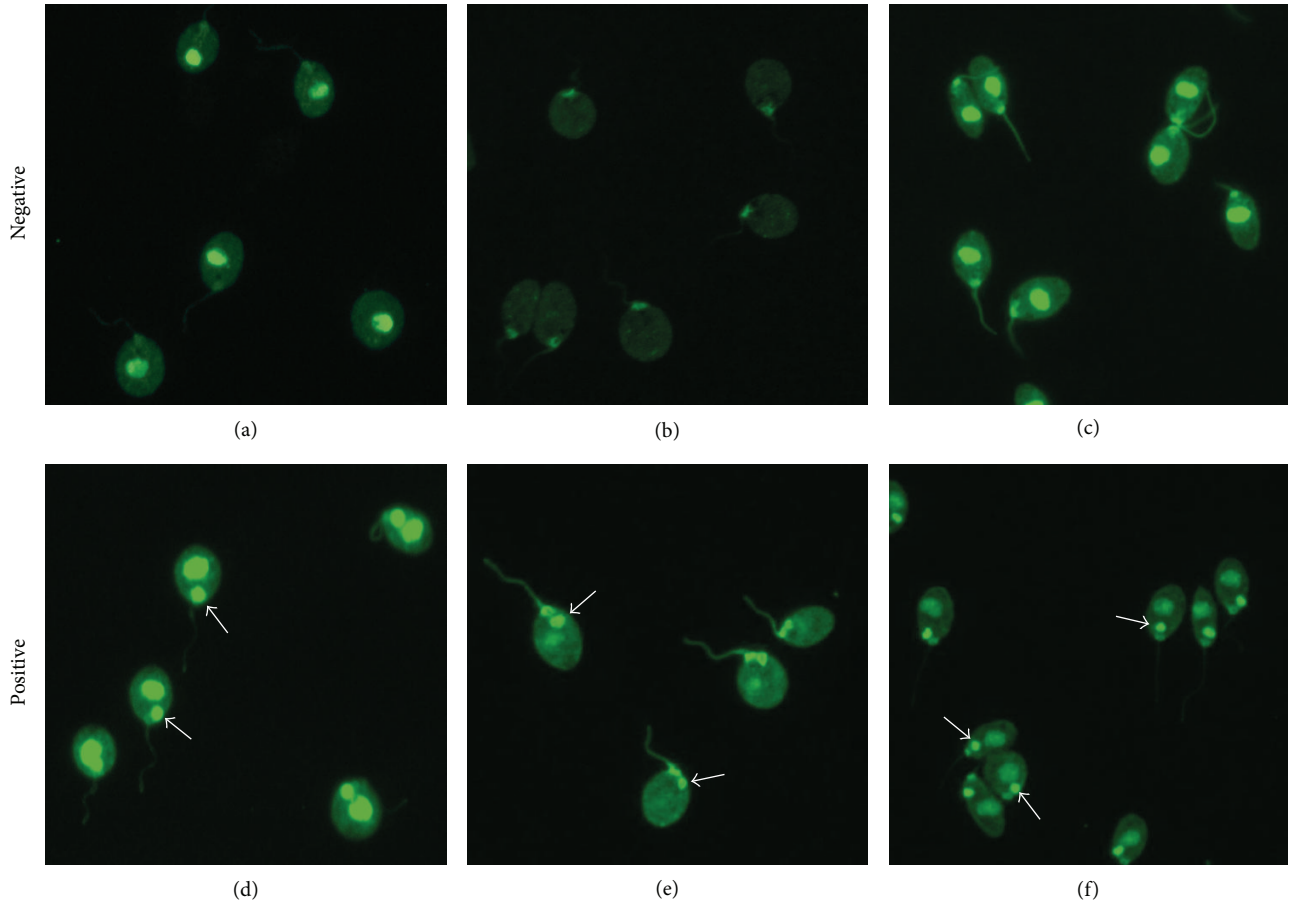

Figure 2: Immunofluorescence patterns on *Crithidia luciliae* revealing the absence or presence of antibodies against dsDNA. Samples are determined as anti-dsDNA negative if the kinetoplasts of the cells do not fluoresce, irrespective of fluorescent nuclei (a), basal bodies (b), or both (c). Samples are determined as anti-dsDNA positive as soon as the kinetoplasts reveal fluorescence signals above a given threshold (see arrows) which may be accompanied by additional fluorescence of the nuclei (d), the basal body (e), or both (f).

of fluorescence results [39]. Several platforms for IIFT automation, which differ in certain features (e.g., throughput, walk-away times, and DNA counterstain) and applications (e.g., pattern classification, number of recognized patterns, recognition of different substrates, and titer estimation), have been developed and launched for commercial use, facilitating and standardizing ANA IIFT diagnostics in numerous laboratories worldwide [38–42]. Efforts though have been majorly focused on the recognition and interpretation of the HEp-2 cell substrate. Only few commercial systems, among these the EUROPattern-Suite, provide the expanded option of automated evaluation of other substrates such as *Crithidia luciliae* [34, 42], which represents the most important IIFT substrate to detect anti-dsDNA in the context of diagnosing SLE. More comprehensive reports on an automated platform to support *Crithidia luciliae* image classification have been published for a noncommercial computer-aided-diagnosis (CAD) system previously [43, 44].

Similarly to the EUROPattern-Suite technology, the presented CAD system applies a multistep classification approach. Since the optical elements of the EUROPattern microscope, camera resolution, optical magnification, and cell density are adjusted to each other with high-precision, a single image is sufficient for accurate classification of one well/biochip. This leads to high performance and sample throughput in routine diagnostics. The CAD system requires classification of three to five images to classify one well, resulting in one additional classification step compared to the EUROPattern-Suite approach (see Figure 1 in this work and Figure 3 in [43]).

A threshold-based preclassification step is included in both system architectures identifying and separating either negative images (CAD) or negative cells (EUROPattern). The cell-based preclassification of the EUROPattern-Suite is robust regarding image artefacts, avoiding inclusion of false positive images into the further classification process. Single cell classification, based on extracted features, is then applied in the CAD as well as in the EUROPattern system to decide whether single cells and finally the image is positive. However, about 8 cells are recorded per image by the CAD system [44] while the average number of cells within an image taken by the EUROPattern-Suite is about 30.

We did not find any information whether a counterstaining of the cells is used by the CAD system. In the case of the EUROPattern-Suite, Evans blue counterstaining is applied for reliable evaluation of image sharpness and for robust cell segmentation. An option of titer prediction, as provided by EUROPattern, has not been described for the CAD system [43, 44].

Within the scope of this study, the performance of the EUROPattern-Suite in daily clinical routine has been validated. 569 consecutive sera which have been submitted to a reference laboratory for routine anti-dsDNA screening and 100 samples from healthy blood donors were examined by CLIFT to determine anti-dsDNA. Automatically generated results by the EUROPattern-Suite were compared to results obtained through classic visual inspection by two independent experts. This comparison revealed a high accuracy of the automated evaluation strategy. In total, 97.2% of the samples were equally classified. An agreement of 91% was previously reported for another commercial system [34]. Rare cases of inaccuracy in our study exclusively concerned the negative class, as determined by the experts' visual examination, which were proposed to be positive by the software (specificity 96.8%). These false positive classifications were primarily caused by an intensive fluorescence of the basal bodies within the analyzed cells, which was misleading for the software. Further efforts are now focused on the elimination of this inaccuracy. Nevertheless, the physician may rely on the system detecting all positive samples (100% sensitivity), meaning negative results require no closer inspection anymore. Within the GUI, these are displayed in a list, sorted by brightness and classification confidence, and can be easily verified in batches directly at the computer screen. Since the physician's verification is required for generation of the final result, this batchwise processing significantly enhances the overall efficiency of the system. The physician can now focus on the necessary review of the positive samples. Regarding these, the software allows inspection of the images as well as corrections (if required) and validation of the results one after another. Misleading result proposals, therefore, should be counterbalanced by the physician's revision.

The results support the idea of the EUROPattern-Suite standardizing and facilitating SLE diagnostics through automation of CLIFT interpretation. The system is less prone to human interpretation error and functions consistently and time-effectively allowing a long walk-away time [42]. At the same time, it reached concordance with experts of CLIFT interpretation in the vast majority of cases tested during this study. All images and proposed results are displayed within the clearly arranged GUI, which is analogously designed to the ANA evaluation screen and likewise incorporated into the superordinate laboratory management software called EUROLabOffice (ELO) [42]. The results may be revised and need to be validated by the physician in a last step to generate an official end result and a diagnosis directly at the computer screen. Thus, the system has the potential to reach 100% accuracy with respect to visual immunofluorescence microscopy.

Beyond that, ELO manages the complete communication between any standard laboratory information system and the different workstations which can be found in a diagnostic laboratory (e.g., ELISA, IIFT, and immunoblot). All results which have been validated by the physician are reported to ELO and will be merged into one concluding report concerning one sample. The report is integrated into the patient's data history which is accessible via ELO. The integrated database harbors enough memory to additionally save worklists and analytical data, for example, IIFT and immunoblot images. Thus, ELO ensures automated data transfer avoiding errors by manual input, increases laboratory efficiency by taking over several organizational tasks, and supersedes the classical laboratory paper archive.

4.1. Perspectives. Further applications of the EUROPattern-Suite are currently under validation or development. These include the already available option of automated immunofluorescence evaluation of antibodies against neutrophil granulocytes (ANCA) using human cell (granulocytes) substrate. The software performs positive/negative classification as well as ANCA pattern discrimination as majorly required in vasculitis diagnostics. Automated documentation and image acquisition of numerous tissues (e.g., liver, kidney, stomach, esophagus, small intestine, heart, and neuronal tissue), which are provided as cryosections on the biochips, is also feasible. With their help, organ as well as nonorgan specific antibodies (e.g., anti-mitochondrial antibodies (AMA), antibodies against epithelial membranes (EMA), epidermal basement membrane or desmosomes, anti-heart muscle-antibodies, or antibodies against various neuronal proteins) may be detected which play an important role in the diagnostics of various other autoimmune diseases.

5. Conclusion

Automated determination of antibodies against dsDNA using the EUROPattern-Suite for computer-aided immunofluorescence microscopy on *Crithidia luciliae* represents a new tool in SLE diagnostics. Validation of this system revealed 100% sensitivity and high specificity (96.8%) for recognition and discrimination of anti-dsDNA positive and negative samples compared to visual inspection by experts of CLIFT evaluation. To raise the system's efficiency, negative samples can be verified in batches while positive samples are individually controlled and verified by an expert. In combination with the superordinate laboratory management system EUROLabOffice, the EUROPattern-Suite, therefore, enables a more standardized interpretation of CLIFT and a reduction of laboratory workload.

Conflict of Interests

Stefan Gerlach, Kai Affeldt, Lena Pototzki, Christopher Krause, Jörn Voigt, Johanna Fraune, and Kai Fechner are employees of EUROIMMUN AG.

Acknowledgments

The authors thank Dr. Konstantin Ens, Melanie Hahn, Maick Danckwardt, and Christian Feirer for their assistance and

participation in the development of the EUROPattern-Suite technology and Dr. Jacqueline Gosink for careful and critical reading of the paper.

References

[1] "Guidelines for referral and management of systemic lupus erythematosus in adults. American College of Rheumatology Ad Hoc Committee on Systemic Lupus Erythematosus Guidelines," *Arthritis & Rheumatism*, vol. 42, no. 9, pp. 1785–1796, 1999.

[2] E. Cozzani, M. Drosera, G. Gasparini, and A. Parodi, "Serology of lupus erythematosus: correlation between immunopathological features and clinical aspects," *Autoimmune Diseases*, vol. 2014, Article ID 321359, 13 pages, 2014.

[3] E. M. Tan, A. S. Cohen, J. F. Fries et al., "The 1982 revised criteria for the classification of systemic lupus erythrematosus," *Arthritis & Rheumatism*, vol. 25, no. 11, pp. 1271–1277, 1982.

[4] M. Petri, A.-M. Orbai, G. S. Alarcõn et al., "Derivation and validation of the systemic lupus international collaborating clinics classification criteria for systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 64, no. 8, pp. 2677–2686, 2012.

[5] A. J. G. Swaak, J. Groenwold, L. A. Aarden, L. W. S. van Eps, and E. W. Feltkamp, "Prognostic value of anti-dsDNA in SLE," *Annals of the Rheumatic Diseases*, vol. 41, no. 4, pp. 388–395, 1982.

[6] E. J. ter Borg, G. Horst, E. J. Hummel, P. C. Limburg, and C. G. M. Kallenberg, "Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in systemic lupus erythematosus. A long-term, prospective study," *Arthritis & Rheumatism*, vol. 33, no. 5, pp. 634–643, 1990.

[7] M. C. J. van Bruggen, C. Kramers, M. N. Hylkema, R. J. T. Smeenk, and J. H. M. Berden, "Significance of anti-nuclear and anti-extracellular matrix autoantibodies for albuminuria in murine lupus nephritis; A longitudinal study on plasma and glomerular eluates in MRL/l mice," *Clinical & Experimental Immunology*, vol. 105, no. 1, pp. 132–139, 1996.

[8] S. Yung and T. M. Chan, "Autoantibodies and resident renal cells in the pathogenesis of lupus nephritis: getting to know the unknown," *Clinical and Developmental Immunology*, vol. 2012, Article ID 139365, 13 pages, 2012.

[9] S. Yung and T. M. Chan, "Anti-DNA antibodies in the pathogenesis of lupus nephritis—the emerging mechanisms," *Autoimmunity Reviews*, vol. 7, no. 4, pp. 317–321, 2008.

[10] W. Egner, "The use of laboratory tests in the diagnosis of SLE," *Journal of Clinical Pathology*, vol. 53, no. 6, pp. 424–432, 2000.

[11] K. H. Kim, J. Y. Han, J. M. Kim, S. W. Lee, and W. T. Chung, "Clinical significance of ELISA positive and immunofluorescence negative anti-dsDNA antibody," *Clinica Chimica Acta*, vol. 380, no. 1-2, pp. 182–185, 2007.

[12] T. R. Chiaro, K. W. Davis, A. Wilson, B. Suh-Lailam, and A. E. Tebo, "Significant differences in the analytic concordance between anti-dsDNA IgG antibody assays for the diagnosis of systemic lupus erythematosus—implications for inter-laboratory testing," *Clinica Chimica Acta*, vol. 412, no. 11-12, pp. 1076–1080, 2011.

[13] A.-M. Rouquette and C. Desgruelles, "Detection of antibodies to dsDNA: an overview of laboratory assays," *Lupus*, vol. 15, no. 7, pp. 403–407, 2006.

[14] K. Haugbro, J. C. Nossent, T. Winkler, Y. Figenschau, and O. P. Rekvig, "Anti-dsDNA antibodies and disease classification in antinuclear antibody positive patients: the role of analytical diversity," *Annals of the Rheumatic Diseases*, vol. 63, no. 4, pp. 386–394, 2004.

[15] R. Biesen, C. Dähnrich, A. Rosemann et al., "Anti-dsDNA-NcX ELISA: dsDNA-loaded nucleosomes improve diagnosis and monitoring of disease activity in systemic lupus erythematosus," *Arthritis Research and Therapy*, vol. 13, no. 1, article R26, 2011.

[16] A. Antico, S. Platzgummer, D. Bassetti, N. Bizzaro, R. Tozzoli, and D. Villalta, "Diagnosing systemic lupus erythematosus: new-generation immunoassays for measurement of anti-dsDNA antibodies are an effective alternative to the Farr technique and the *Crithidia luciliae* immunofluorescence test," *Lupus*, vol. 19, no. 8, pp. 906–912, 2010.

[17] B. B. Suh-Lailam, T. R. Chiaro, K. Wayne Davis, A. R. Wilson, and A. E. Tebo, "Evaluation of a high avidity anti-dsDNA igG enzyme-linked immunosorbent assay for the diagnosis of systemic lupus erythematosus," *International Journal of Clinical and Experimental Pathology*, vol. 4, no. 8, pp. 748–754, 2011.

[18] R. J. T. Smeenk, "Detection of antibodies to dsDNA: current insights into its relevance," *Clinical and Experimental Rheumatology*, vol. 20, no. 3, pp. 294–300, 2002.

[19] H. C. Nossent and O. P. Rekvig, "Is closer linkage between systemic lupus erythematosus and anti-double-stranded DNA antibodies a desirable and attainable goal?" *Arthritis Research and Therapy*, vol. 7, no. 2, pp. 85–87, 2005.

[20] R. Tozzoli, N. Bizzaro, E. Tonutti et al., "Guidelines for the laboratory use of autoantibody tests in the diagnosis and monitoring of autoimmune rheumatic diseases," *American Journal of Clinical Pathology*, vol. 117, no. 2, pp. 316–324, 2002.

[21] L. A. Aarden, E. R. de Groot, and T. E. W. Feltkamp, "Immunology of DNA. III. Crithidia luciliae, a simple substrate for the determination of anti-dsDNA with the immunofluorescence technique," *Annals of the New York Academy of Sciences*, vol. 254, pp. 505–515, 1975.

[22] N. Bizzaro, R. Tozzoli, E. Tonutti et al., "Variability between methods to determine ANA, anti-dsDNA and anti-ENA autoantibodies: a collaborative study with the biomedical industry," *Journal of Immunological Methods*, vol. 219, no. 1-2, pp. 99–107, 1998.

[23] D. Roggenbuck, D. Reinhold, R. Hiemann, U. Anderer, and K. Conrad, "Standardized detection of anti-ds DNA antibodies by indirect immunofluorescence—a new age for confirmatory tests in SLE diagnostics," *Clinica Chimica Acta*, vol. 412, no. 21-22, pp. 2011–2012, 2011.

[24] A. Rigon, P. Soda, D. Zennaro, G. Iannello, and A. Afeltra, "Indirect immunofluorescence in autoimmune diseases: assessment of digital images for diagnostic purpose," *Cytometry Part B: Clinical Cytometry*, vol. 72, no. 6, pp. 472–477, 2007.

[25] M. J. Fritzler, "The antinuclear antibody test: last or lasting gasp?" *Arthritis and Rheumatism*, vol. 63, no. 1, pp. 19–22, 2011.

[26] U. Sack, S. Knoechner, H. Warschkau, U. Pigla, F. Emmrich, and M. Kamprad, "Computer-assisted classification of HEp-2 immunofluorescence patterns in autoimmune diagnostics," *Autoimmunity Reviews*, vol. 2, no. 5, pp. 298–304, 2003.

[27] R. Hiemann, T. Büttner, T. Krieger, D. Roggenbuck, U. Sack, and K. Conrad, "Challenges of automated screening and differentiation of non-organ specific autoantibodies on HEp-2 cells," *Autoimmunity Reviews*, vol. 9, no. 1, pp. 17–22, 2009.

[28] American College of Rheumatology, *American College of Rheumatology Position Statement: Methodology of Testing for Antinuclear Antibodies*, 2009, http://www.rheumatology.org.

[29] W. Stöcker and W. Schlumberger, "Antinukleäre Antikörper," in *Lexikon der Medizinischen Laboratoriumsdiagnostik*, A. M. Gressner and T. Arndt, Eds., vol. 2, Springer Medizin, Heidelberg, Germany, 2013.

[30] N. Agmon-Levin, J. Damoiseaux, C. Kallenberg et al., "International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies," *Annals of the Rheumatic Diseases*, vol. 73, no. 1, pp. 17–23, 2014.

[31] K. Egerer, D. Roggenbuck, R. Hiemann et al., "Automated evaluation of autoantibodies on human epithelial-2 cells as an approach to standardize cell-based immunofluorescence tests," *Arthritis Research & Therapy*, vol. 12, no. 2, article R40, 2010.

[32] J. Voigt, C. Krause, E. Rohwäder et al., "Automated indirect immunofluorescence evaluation of antinuclear autoantibodies on HEp-2 cells," *Clinical and Developmental Immunology*, vol. 2012, Article ID 651058, 7 pages, 2012.

[33] S. Kivity, B. Gilburd, N. Agmon-Levin et al., "A novel automated indirect immunofluorescence autoantibody evaluation," *Clinical Rheumatology*, vol. 31, no. 3, pp. 503–509, 2012.

[34] A. Melegari, C. Bonaguri, A. Russo, B. Luisita, T. Trenti, and G. Lippi, "A comparative study on the reliability of an automated system for the evaluation of cell-based indirect immunofluorescence," *Autoimmunity Reviews*, vol. 11, no. 10, pp. 713–716, 2012.

[35] C. Bonroy, C. Verfaillie, V. Smith et al., "Automated indirect immunofluorescence antinuclear antibody analysis is a standardized alternative for visual microscope interpretation," *Clinical Chemistry and Laboratory Medicine*, vol. 51, no. 9, pp. 1771–1779, 2013.

[36] X. Bossuyt, S. Cooreman, H. de Baere et al., "Detection of antinuclear antibodies by automated indirect immunofluorescence analysis," *Clinica Chimica Acta*, vol. 415, pp. 101–106, 2013.

[37] S. S. Copple, T. D. Jaskowski, R. Giles, and H. R. Hill, "Interpretation of ANA indirect immunofluorescence test outside the darkroom using NOVA view compared to manual microscopy," *Journal of Immunology Research*, vol. 2014, Article ID 149316, 7 pages, 2014.

[38] R. Tozzoli, A. Antico, B. Porcelli, and D. Bassetti, "Automation in indirect immunofluorescence testing: a new step in the evolution of the autoimmunology laboratory," *Autoimmunity Highlights*, vol. 3, no. 2, pp. 59–65, 2012.

[39] R. Tozzoli, C. Bonaguri, A. Melegari, A. Antico, D. Bassetti, and N. Bizzaro, "Current state of diagnostic technologies in the autoimmunology laboratory," *Clinical Chemistry and Laboratory Medicine*, vol. 51, no. 1, pp. 129–138, 2013.

[40] N. Bizzaro, A. Antico, S. Platzgummer et al., "Automated antinuclear immunofluorescence antibody screening: a comparative study of six computer-aided diagnostic systems," *Autoimmunity Reviews*, vol. 13, no. 3, pp. 292–298, 2014.

[41] P. L. Meroni, N. Bizzaro, I. Cavazzana, M. O. Borghi, and A. Tincani, "Automated tests of ANA immunofluorescence as throughput autoantibody detection technology: strengths and limitations," *BMC Medicine*, vol. 12, no. 1, article 38, 2014.

[42] C. Krause, K. Ens, K. Fechner et al., "EUROPattern-Suite technology for computer-aided immunofluorescence microscopy in autoantibody diagnostics," *Lupus*, vol. 24, no. 4-5, pp. 516–529, 2015.

[43] P. Soda, L. Onofri, and G. Iannello, "A decision support system for Crithidia Luciliae image classification," *Artificial Intelligence in Medicine*, vol. 51, no. 1, pp. 67–74, 2011.

[44] F. Buzzulini, A. Rigon, P. Soda et al., "The classification of Crithidia luciliae immunofluorescence test (CLIFT) using a novel automated system," *Arthritis Research and Therapy*, vol. 16, no. 2, article R71, 2014.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 369462, 15 pages
http://dx.doi.org/10.1155/2015/369462

Research Article

Nonbilayer Phospholipid Arrangements Are Toll-Like Receptor-2/6 and TLR-4 Agonists and Trigger Inflammation in a Mouse Model Resembling Human Lupus

Carlos Wong-Baeza,[1,2] Alonso Tescucano,[1] Horacio Astudillo,[3] Albany Reséndiz,[1] Carla Landa,[1] Luis España,[1] Jeanet Serafín-López,[4] Iris Estrada-García,[4] Sergio Estrada-Parra,[4] Leopoldo Flores-Romo,[2] Carlos Wong,[1] and Isabel Baeza[1]

[1] *Biochemistry Department, National School of Biological Sciences, National Polytechnic Institute (IPN), 11340 Mexico City, DF, Mexico*
[2] *Cell Biology Department, Center for Research and Advanced Studies of the National Polytechnic Institute (IPN), 07360 Mexico City, DF, Mexico*
[3] *Oncology Department, Mexican Institute of Social Security, XXI Century National Medical Center, 06720 Mexico City, DF, Mexico*
[4] *Immunology Department, National School of Biological Sciences, National Polytechnic Institute (IPN), 11340 Mexico City, DF, Mexico*

Correspondence should be addressed to Isabel Baeza; isabelbaeza@yahoo.com

Received 2 April 2015; Revised 1 June 2015; Accepted 6 July 2015

Academic Editor: Lingyun Sun

Systemic lupus erythematosus is characterized by dysregulated activation of T and B cells and autoantibodies to nuclear antigens and, in some cases, lipid antigens. Liposomes with nonbilayer phospholipid arrangements induce a disease resembling human lupus in mice, including IgM and IgG antibodies against nonbilayer phospholipid arrangements. As the effect of these liposomes on the innate immune response is unknown and innate immune system activation is necessary for efficient antibody formation, we evaluated the effect of these liposomes on Toll-like receptor (TLR) signaling, cytokine production, proinflammatory gene expression, and T, NKT, dendritic, and B cells. Liposomes induce TLR-4- and, to a lesser extent, TLR-2/TLR-6-dependent signaling in TLR-expressing human embryonic kidney (HEK) cells and bone marrow-derived macrophages. Mice with the lupus-like disease had increased serum concentrations of proinflammatory cytokines, C3a and C5a; they also had more TLR-4-expressing splenocytes, a higher expression of genes associated with TRIF-dependent TLR-4-signaling and complement activation, and a lower expression of apoptosis-related genes, compared to healthy mice. The percentage of NKT and the percentage and activation of dendritic and B2 cells were also increased. Thus, TLR-4 and TLR-2/TLR-6 activation by nonbilayer phospholipid arrangements triggers an inflammatory response that could contribute to autoantibody production and the generation of a lupus-like disease in mice.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a loss of tolerance to nuclear antigens and by dysregulated activation of T and B cells. Polyclonal activation of B cells leads to the production of large quantities of autoreactive antibodies and the formation of immune complexes, which causes tissue damage. In some SLE patients, it has been shown that bone marrow mesenchymal stem cells exhibit impaired capacities for proliferation, differentiation, migration [1], and immune modulation [2]. Genetic defects, drug exposure, infectious agents, and environmental factors can also contribute to the pathogenesis of this disease [3, 4]. SLE has an incidence in Europe and North America of approximately 10 cases per 100,000 population per year, and it is estimated that 10% of these cases are drug-induced. Drug-induced lupus erythematosus (DILE) is a lupus-like syndrome that resolves upon drug discontinuation. The drugs more frequently associated with the induction of this lupus-like syndrome are procainamide (antiarrhythmic), hydralazine (antihypertensive), and chlorpromazine (antipsychotic) [5, 6].

Animal models of SLE include lupus-prone mice, which spontaneously develop lupus, and normal mice that develop lupus after injection of lymphocytes from lupus-prone mice, immunization with prototypical lupus antigens (DNA- and RNA-protein complexes), or injection of pristane (2,6,10,14-tetramethylpentadecane) [3, 7]. The most commonly used lupus-prone mice are the F_1 hybrids of New Zealand black (NZB) and NZ white (NZB/NZW F_1) mice, the Murphy-Roths large/lymphoproliferative locus (MLR/lpr) mice, and the recombinant C57BL/6 female and SB/Le male strain/Y-linked autoimmune accelerator (BXSB/Yaa) mice [3, 8, 9]. Our group has also developed a mouse model of autoimmune disease resembling human lupus that can be induced in normal mice [10]. In this model, the disease is triggered by liposomes with nonbilayer phospholipid arrangements. Liposomes are model membranes made of cylindrical phospholipids, such as phosphatidylcholine, and H_{II}-preferring (conical shaped) phospholipids, such as phosphatidic acid, phosphatidylserine, or cardiolipin [11]. Conical phospholipids can form molecular associations distinct to lipid bilayers, known as nonbilayer phospholipid arrangements, in the presence of inducers such as Mn^{2+} [12, 13] or the drugs chlorpromazine and procainamide, which can trigger DILE in humans [10]. Nonbilayer phospholipid arrangements are formed by an inverted micelle (made of conical phospholipids with their polar heads towards the center of the micelle, where the inducer is also located) inserted into and distorting the shape of the phospholipid bilayer (Figure 1(a)). We demonstrated that liposomes with nonbilayer phospholipid arrangements induced by Mn^{2+}, chlorpromazine, or procainamide cause an autoimmune disease resembling human lupus in mice. A similar disease is produced by treating mice directly with Mn^{2+}, chlorpromazine, or procainamide (which induce nonbilayer phospholipid arrangements on mouse cells) or by injecting the monoclonal antibody H308 (which binds specifically to nonbilayer phospholipid arrangements and stabilizes these arrangements on mouse cells) [10, 14].

IgM and IgG antibodies against nonbilayer phospholipid arrangements are found in the sera of mice with the autoimmune disease resembling human lupus, and also in the sera of patients with lupus [10, 15]. Usually, the efficient production of IgG antibodies requires an activation of the innate immune response. Therefore we hypothesized that nonbilayer phospholipid arrangements could be Toll-like receptor- (TLR-) 4/MD-2 agonists, as their molecular structure is similar to that of the lipid A from bacterial lipopolysaccharide (LPS). Lipid A is formed by a β-1,6-D-glucosamine disaccharide with two (negatively charged) phosphates and six saturated acyl chains in an asymmetric distribution (four chains are bound to the nonreducing and two to the reducing glucosamine). Hexaacylated asymmetric lipid A molecules have a conical molecular shape, because the cross section of the hydrophobic region is larger than that of the hydrophilic region (Figure 1(b)). Hexaacylated symmetric lipid A (with three acyl chains bound to the nonreducing and three to the reducing glucosamine) and penta- and tetraacylated lipid A molecules have a cylindrical molecular shape, and they do not have biological activity [16, 17]. The intrinsic conformation of lipid A is not altered when saccharide groups are added, as in LPS. The LPS molecules form multimeric aggregates in water: if the lipid A is cylindrical, they form a smooth bilayer arrangement, but conical lipid A molecules form a nonbilayer or hexagonal (H_{II}) arrangement [17]. LPS-binding protein (LBP) is a plasma protein that facilitates the transfer of LPS molecules from these hexagonal (H_{II}) arrangements to CD14, and membrane-bound CD14 delivers LPS to TLR-4/MD2 [18]. Since the conical molecular shape of lipid A is a requirement for TLR-4/MD-2 triggering [16–19], we hypothesized that liposomes with nonbilayer phospholipid arrangements, but not smooth liposomes (with phospholipids in a bilayer arrangement), could trigger TLR-4/MD-2 signaling.

In this study, we investigated whether liposomes with nonbilayer phospholipid arrangements are TLR-4/MD-2 agonists, because the activation of this innate immune receptor leads to the production of proinflammatory cytokines. We also looked for proinflammatory cytokines in the sera of mice with the autoimmune disease triggered by liposomes with nonbilayer phospholipid arrangements, and we determined the gene expression profile in the spleens of these mice, focusing on the expression of proinflammatory genes. In addition, we determined the relative percentage and activation of T, NKT, dendritic, and B cells in the spleen of mice with the disease. This study contributes to the understanding of the pathological and genetic features of a novel mouse model of human lupus.

2. Materials and Methods

2.1. Preparation and Characterization of Liposomes. Egg-yolk L-α-phosphatidic acid, bovine brain L-α-phosphatidylserine, egg-yolk L-α-phosphatidylcholine, chlorpromazine, procainamide, and chloroquine were purchased from Sigma (St. Louis, MO, USA). Liposomes contained the cylindrical shaped phospholipid phosphatidylcholine and a conical phospholipid (phosphatidic acid or phosphatidylserine). The molar ratios (phosphatidylcholine/phosphatidic acid 2 : 1, phosphatidylcholine/phosphatidylserine 4 : 1) were optimized for the induction of nonbilayer phospholipid arrangements [14]. Nine micromoles of phospholipid mixture was dissolved in 1 mL diethyl ether and 330 μL of TS buffer (10 mM Tris-HCl, 1 mM NaCl, pH 7), mixed and sonicated three times in a G112SPI sonicator (Laboratory Supplies, Hicksville, NY, USA). The diethyl ether was then removed under a stream of oxygen-free dry nitrogen at reduced pressure, using a rotary evaporator at 37°C. The liposomes were filtered through 0.45 μm MF-Millipore membranes (Billerica, MA, USA) to homogenize their size.

To induce the formation of nonbilayer phospholipid arrangements, liposomes in TS buffer were incubated for 30 min at 37°C in the presence of 0.5–4 mM $MnCl_2$, 0.5–3 mM chlorpromazine, and 4–32 mM procainamide [14]. All of the final preparations of liposomes were negative for LPS contamination, as assessed by the gel clot LAL method (Charles River Endosafe, Charleston, SC, USA).

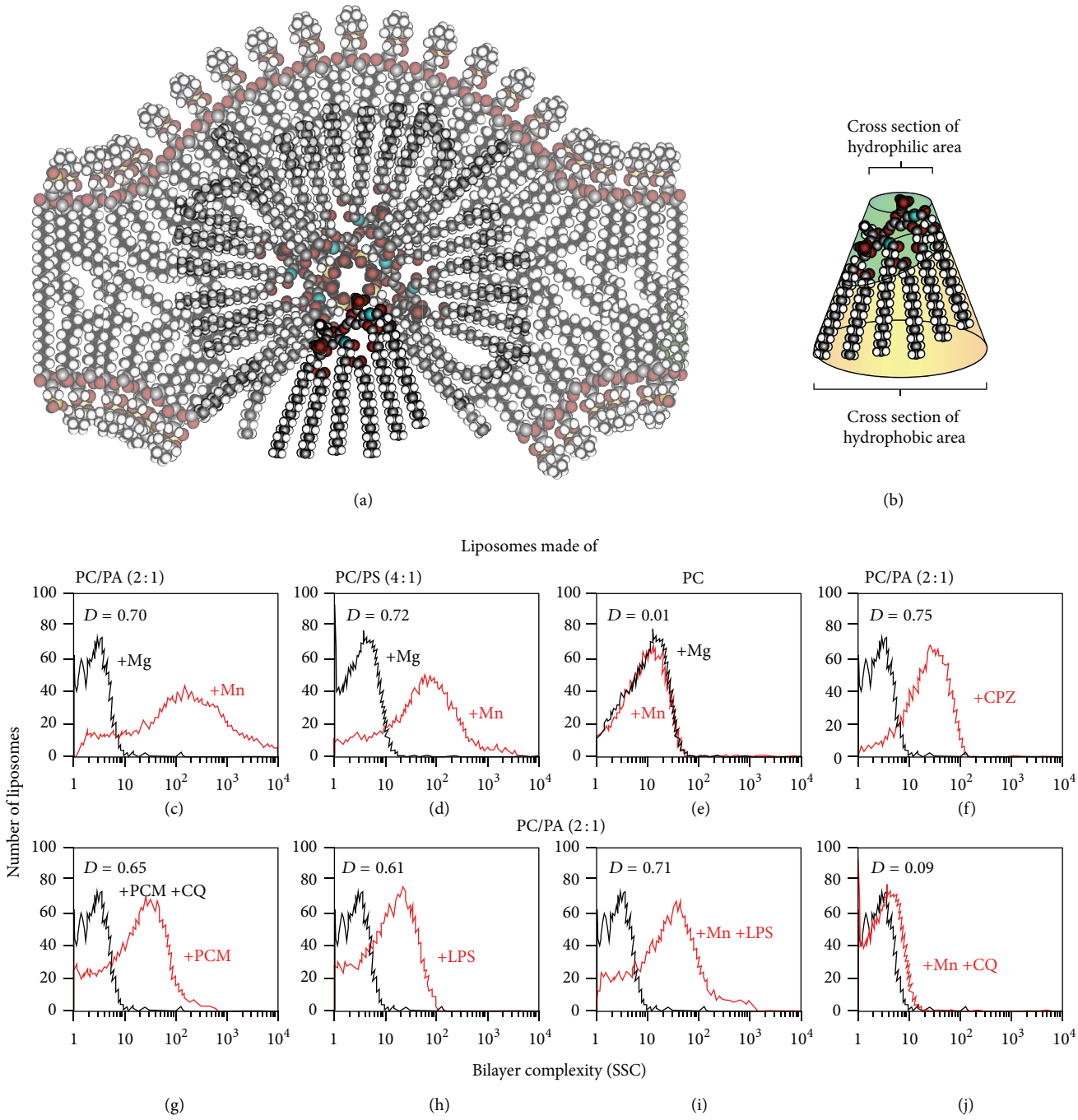

FIGURE 1: Structure and characterization of nonbilayer phospholipid arrangements. (a) Representation of a nonbilayer phospholipid arrangement, showing an inverted micelle, with the acyl chains of the phospholipids in a conical arrangement, inserted into the lipid bilayer. (b) Molecular shape of the lipid A of LPS, showing the conical arrangement of its acyl chains. (c to j) Liposomes made of egg-yolk phosphatidylcholine (PC)/egg-yolk phosphatidic acid (PA) (2 : 1 molar ratio), PC/bovine brain phosphatidylserine (PS) (4 : 1) (50 nmol anionic phospholipid in 50 μL TS buffer), or PC alone were incubated at 37°C for 30 min with 2 mM $MnCl_2$, 1.5 mM chlorpromazine (CPZ), 8 mM procainamide (PCM), 100 ng/mL LPS, 0.1 mM chloroquine (CQ), 0.2 mM $MgCl_2$, or the indicated mixtures of cations or compounds, respectively. Changes in bilayer complexity (SSc) are represented as histograms: black lines represent liposomes alone or incubated with Mg or PCM + CQ; red lines represent liposomes incubated with Mn or the indicated compound. Values of Kolmogorov-Smirnov test of $D \geq 0.5$, $p < 0.001$, indicate a significant difference between the compared histograms. A representative experiment of five is shown.

The detection of nonbilayer phospholipid arrangements by flow cytometry was previously validated by freeze-fracture electron microscopy and ^{31}P-NMR spectroscopy [10, 14, 15]. Therefore, in this study we only used flow cytometry to demonstrate the formation of these arrangements on liposomes. Liposomes and liposomes with nonbilayer phospholipid arrangements in TS buffer were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with CellQuest software. Ten thousand events were acquired for each sample.

2.2. TLR Activation Assays. Human embryonic kidney (HEK) 293 cells, nontransfected or stably transfected with human TLR-4/MD2/CD14, TLR-2/TLR-6, TLR-5, or TLR-8, were purchased from InvivoGen (San Diego, CA, USA). The expression of the TLRs was verified by flow cytometry. The HEK-TLR transfectants were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 10 μg/mL blasticidin (InvivoGen), and 100 μg/mL normocin (InvivoGen). HygroGold (25 μg/mL; InvivoGen) was also added to the media of the HEK-TLR-4/MD2/CD14 cell line. The viability of these cell lines in the presence of Mn^{2+}, chlorpromazine, procainamide, or chloroquine, and in the presence of liposomes or liposomes with nonbilayer phospholipid arrangements, was evaluated with the Alamar Blue method [20].

To assess TLR activation, the cell lines were incubated in the presence of liposomes made of phosphatidylcholine/phosphatidic acid (2 : 1), alone or with nonbilayer phospholipid arrangements induced by Mn^{2+} (2–4 mM). As a negative control, the liposomes with nonbilayer arrangements were previously incubated with 0.1 mM chloroquine. For the positive controls, the cell lines were incubated in the presence of their known TLR agonists: 100 ng/mL *Escherichia coli* 0111:B4 LPS for HEK-TLR-4/MD2/CD14, 1 μg/mL FSL-1 (a synthetic lipoprotein derived from *Mycoplasma salivarium*) for HEK-TLR-2/TLR-6, 1 μg/mL *Salmonella typhimurium* flagellin for HEK-TLR-5, and 2.5 μg/mL ssRNA40 (a 20 mer phosphorothioate-protected single-stranded RNA oligonucleotide containing a GU-rich sequence) for HEK-TLR-8. All TLR agonists were sourced from InvivoGen. After 24 h, the cell culture supernatants were harvested and assayed for IL-8 production (BD OptEIA Set Human IL-8, BD Biosciences, San Diego, CA, USA). NF-κB activation was assayed in cell culture extracts using the reporter plasmid pNiFty-Luc (Promega Corporation, Madison, WI, USA).

In order to determine if chloroquine affects the viability of HEK293 cells, the LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen) was used. HEK293 cells were incubated with 0.05, 0.1, and 0.5 mM of chloroquine for 24 h at 37°C and 5% CO_2. The cells were then transferred to a tube and stained with 50 μL of LIVE/DEAD diluted 1 : 100 in distilled water and incubated for 15 min at room temperature in the dark. FACS lysis buffer (1 mL; Becton Dickinson) was added for erythrocyte lysis, and the cells were incubated for 10 min at room temperature in the dark. The cells were washed with 2 mL of phosphate-buffered saline (PBS) and resuspended in 300 μL of PBS and analyzed by flow cytometry. Forty thousand events were acquired for each sample with a LSR Fortessa cytometer (Becton-Dickinson).

To evaluate whether chloroquine can induce apoptosis of HEK293 cells, the Annexin V-propidium iodide staining method was used. HEK293 cells were incubated with 0.05, 0.1, and 0.5 mM of chloroquine for 24 h at 37°C and 5% CO_2. The cells were then transferred to a tube and washed with 1 mL of Annexin V-binding buffer (eBioscience, San Diego, CA, USA). 100 μL of 2 μg/mL Annexin V-APC (eBioscience) in Annexin V-binding buffer was added, and the cells were incubated for 15 min at room temperature in the dark. The cells were washed with 1 mL of Annexin V-binding buffer, resuspended in 100 μL of the same buffer containing 1 μg of propidium iodide (BioLegend, San Diego CA, USA) and incubated for 15 min at room temperature in the dark. The cells were washed and resuspended in the Annexin V-binding buffer and analyzed immediately by flow cytometry. Forty thousand events were acquired for each sample in a LSR Fortessa cytometer (Becton-Dickinson).

TLR stimulation was also analyzed in bone marrow-derived macrophages (BMDM) from BALB/c mice. BMDM were obtained from the femur and shinbone of female 2-month-old BALB/c mice and they were cultured in RPMI media with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin (Gibco), 50 μg/mL streptomycin (Gibco), and 10 ng/mL recombinant M-CSF (BioLegend) for 7 days. For TLR stimulation, the BMDM were cultured in 96-well plates and stimulated with 10 ng/mL LPS, 1 μg/mL peptidoglycan (PGN; InvivoGen), or 10 or 20 μL of smooth liposomes or liposomes bearing nonbilayer phospholipids arrangements, respectively. After incubation for 24 h at 37°C, the supernatants were collected and analyzed by enzyme-linked immunoabsorbent assay (ELISA; BioLegend) for tumor necrosis factor- (TNF-) α production. For the blocking experiments, 10 μg/mL of anti-TLR-2 (clone T2.5, BioLegend) or 20 μg/mL of anti-TLR-4 (clone MTS510, BioLegend) was added 2 h before the liposomes. The cells were incubated for 24 h at 37°C and the supernatants were collected and analyzed for TNF-α. IgG1,κ (clone MOPC-21, BioLegend) and IgG2a,κ (clone RTK2758, BioLegend) isotype controls were used for the blocking antibodies.

2.3. Mouse Model of Autoimmune Disease Resembling Human Lupus. Forty female 2-month-old specific-pathogen-free BALB/c mice were divided into four groups. The first and the second groups were injected intrasplenically, on days 1 and 15, with phosphatidylcholine/phosphatidic acid (2 : 1) liposomes that had been incubated with 5 mM $MnCl_2$ (Mn group) or 3 mM chlorpromazine (CPZ group). Mice received the same amount of liposomes by intraperitoneal injection on day 30 and then every week for 6 months [10]. The negative control groups consisted of 10 mice treated in the same way but using TS buffer alone (Control group I), or liposomes made of phosphatidylcholine/phosphatidic acid (2 : 1) alone (Control group II).

Blood was taken from mice before liposome injection and each month after the first intraperitoneal injection, for a total

of 6 months. Sera were heated at 56°C for 30 min to inactivate complement and frozen in aliquots at −70°C. To confirm that these mice developed the disease resembling human lupus, we measured anti-nonbilayer phospholipid arrangements, anti-cardiolipin, anti-histone, and anti-coagulant antibodies in their sera. Anti-nonbilayer phospholipid arrangements antibodies were measured by ELISA where the wells were coated with liposomes with or without nonbilayer phospholipid arrangements [14]. Anti-cardiolipin and anti-histone antibodies were also measured by ELISA. Results are reported as arbitrary units (AU) calculated as (AsP − AsW)/(AsH − AsW), where AsP is the absorbance obtained with the sera of mice injected with the liposomes, AsH is the absorbance obtained with the sera of mice before the injection of liposomes, and AsW is absorbance of controls without sera [10]. A modification of the kaolin-activated thromboplastin time test was used to determine the anti-coagulant antibodies; results are reported as the coagulation time in seconds [21].

Three mice from each of the four groups indicated above were euthanized 4 months after the first injection of nonbilayer phospholipid arrangements, when they had the highest titers of anti-nonbilayer phospholipid arrangements, anti-cardiolipin, anti-histone, and anti-coagulant antibodies, and their spleens were used for gene and protein expression studies. The experimental protocols for animal care and use were reviewed and approved by the Bioethics Committee of our Institution according to the "Guide for the Care and Use of Laboratory Animals," which was published by the US National Institute of Health [22].

2.4. Quantification of Cytokines in Mouse Sera. The serum concentrations of interleukin-6 (IL-6), IL-10, IL-12p70, interferon-γ (IFN-γ), TNF-α, and monocyte chemoattractant protein- (MCP-) 1 were measured with a bead-based multiplex immunoassay (BD CBA Mouse Inflammation Kit). Data were acquired with a FACSCalibur flow cytometer, with CellQuest software.

2.5. Evaluation of Gene and Protein Expression in Mouse Spleens. Mouse spleens were sectioned and placed in two cryotubes, one with RNAlater (Invitrogen) for RNA expression studies and one with Tissue-Tek (Sakura Finetek, Torrance, CA, USA) for protein analysis. The cryotubes were stored at −70°C until use. To isolate RNA, the tissue stored in RNAlater was thawed and disaggregated at 15,000 rpm with a TissueRuptor (Qiagen, Valencia, CA, USA), and total RNA was extracted from the tissue homogenates using an RNeasy Mini Kit (Qiagen). The quality and quantity of the RNA samples were assessed in an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA, USA) and a NanoDrop 2000 (Thermo Fisher Scientific, Auburn, AL, USA), respectively; only RNA samples with a RNA integrity number (RIN) ≥ 7 were used for the gene expression analysis.

Total RNA (400 ng) was amplified and labeled using the Quick Amp Labeling Kit (Agilent), and the cyanine-3- or cyanine-5-labeled cRNA was purified with an RNeasy Mini Kit (Qiagen). The cRNA were hybridized to 4×44 K whole mouse genome microarray chips (Agilent, G4122F); the microarrays were scanned with an Agilent Microarray scanner (G2565BA) and the data were extracted with Agilent Feature Extraction software (v.9.5.3.1). Normalization was performed with GeneSpring GX 11.0 software (Agilent). The cutoff for over- and underexpressed genes was set at a mean fold change $\log_2$ ratio greater than +2 or lower than −2, as assessed by two-way analysis of variance (ANOVA; Partek Pro software, Partek Inc., St. Charles, MO, USA) with $p < 0.01$ [23].

To evaluate protein expression, the spleen samples stored in Tissue-Tek were thawed, rinsed with PBS, and disaggregated at 15,000 rpm with a TissueRuptor (Qiagen). The homogenates were centrifuged at 5,000 ×g for 5 min at 4°C, and the supernatants were used to measure C3 (ELISA Kit MBS700250, MyBioSource, San Diego, CA, USA), C5 (ELISA Kit MBS704792, MyBioSource), C3a (ELISA Kit MBS70381, MyBioSource), C5a (ELISA Kit MBS700538, MyBioSource), and IFN-β (ELISA Kit 439407, BioLegend).

TLR-4 was measured by flow cytometry in cells obtained from fresh spleens, which were disaggregated and passed through a 70 μm nylon mesh. The cells were labeled with a fluorescein isothiocyanate- (FITC-) conjugated anti-F4/80 antibody (BioLegend), a PE-conjugated rat anti-mouse TLR-4 antibody (BioLegend), and Fixable Viability Dye 450 (eBiosciences) and acquired in a FACSCalibur flow cytometer. Single viable cells were analyzed, and the percentage of $F4/80^+$ $TLR\text{-}4^+$ cells of the total live cells was determined.

2.6. Evaluation of T, NKT, Dendritic, and B Cells in Mouse Spleens. The spleens of three mice from the groups injected intrasplenically with liposomes without nonbilayer phospholipid arrangements or with liposomes bearing nonbilayer phospholipid arrangements were placed in fluorescence-activated cell sorting (FACS) buffer containing 0.1% BSA and 0.01% sodium azide (Sigma Aldrich). Spleens were disaggregated and passed through a 70 μm nylon mesh. Red blood cells were lysed and spleen cells were resuspended in FACS buffer. Before staining, cells were incubated with Universal Blocking Reagent (Block Biogenex, San Ramón, CA, USA) in PBS for 10 min at 4°C and then washed.

Splenocyte suspensions were labeled with anti-CD19-APC, anti-CD5-AF488, anti-CD69-PerCP, and anti-TLR4-PE (eBioscience) to evaluate B cells; with anti-CD3-FITC, anti-CD4-PE (eBioscience), anti-CD8-APC, and anti-CD69-PerCP to evaluate T cells; with anti-Gr-1-PerCP, anti-CD11c-APC, anti-MHC-II-PE (eBioscience), anti-CD80-FITC, and anti-CD86-PE/Cy7 (eBioscience) to evaluate dendritic cells; and with anti-CD3-FITC and anti-NK 1.1-APC to evaluate NKT cells. Fixable Viability Dye 450 (eBioscience) was added in all cases. The cells were incubated for 30 min at 4°C, then washed with FACS buffer, and fixed with 1% paraformaldehyde (Sigma Aldrich). Labeled cells were acquired in a LSR Fortessa flow cytometer (Becton Dickinson); single, viable cells were analyzed with FlowJo 10.0.6 (Tree Star, Inc., Ashland, OR, USA). Appropriate isotype controls were included in all sets of experiments. All antibodies were from BioLegend unless otherwise indicated.

3. Results

3.1. Lipopolysaccharide Increases the Complexity of Liposomes. We had previously shown that the presence of nonbilayer phospholipid arrangements can be detected by flow cytometry as an increase in side scatter (SSC) value [10, 14, 15]. Thus, the increase in SSC signal after the addition of Mn^{2+}, chlorpromazine, or procainamide to liposomes made of phosphatidylcholine/phosphatidic acid or phosphatidylcholine/phosphatidylserine indicated the presence of nonbilayer phospholipid arrangements (Figures 1(c)-1(d) and 1(f)-1(g)).

As a negative control, we added 5 mM of Mg^{2+} to liposomes (Figures 1(c), 1(d), and 1(e)); Mg^{2+} does not induce the formation of nonbilayer phospholipid arrangements, as was previously shown for phosphatidylcholine/phosphatidic acid liposomes [15]. Liposomes made of the cylindrical lipid phosphatidylcholine, without any conical lipid, did not increase in complexity in the presence of Mn^{2+} or Mg^{2+} (Figure 1(e)), chlorpromazine, or procainamide (data not shown). The addition of LPS caused an increase in SSC signal when it was used alone (Figure 1(h)) or in combination with Mn^{2+} (Figure 1(i)), which suggests that LPS modifies the lipid bilayer. The addition of 0.1 mM chloroquine, a drug that blocks or reverses the formation of nonbilayer phospholipid arrangements [14], decreased the liposome complexity induced by procainamide or Mn^{2+} (Figures 1(g)-1(j)) or chlorpromazine (data not shown).

3.2. Liposomes with Mn^{2+}-Induced Nonbilayer Phospholipid Arrangements Are Toll-Like Receptor- (TLR-) 2/6 and TLR-4 Agonists. We evaluated the effects of phosphatidylcholine/phosphatidic acid liposomes and phosphatidylcholine/phosphatidylserine liposomes, alone or in the presence of Mn^{2+}, chlorpromazine, or procainamide, on the viability of HEK, HEK-TLR-4/MD2/CD14, HEK-TLR-2/TLR-6, HEK-TLR-5, and HEK-TLR-8 cell lines. We found that liposomes made of phosphatidylcholine/phosphatidic acid with Mn^{2+}-induced nonbilayer phospholipid arrangements had no effect on the cell viability (90% or more of cells were viable). These liposomes were then used for TLR activation assays.

Liposomes with Mn^{2+}-induced nonbilayer phospholipid arrangements stimulated IL-8 production by HEK-TLR-4/MD2/CD14 cells and, to a lesser degree, by HEK-TLR-2/TLR-6 cells, but not by HEK-TLR-5 or HEK-TLR-8 cells (Figure 2(a)). Liposomes without nonbilayer phospholipid arrangements or liposomes in which Mn^{2+}-induced nonbilayer phospholipid arrangements had been reversed by chloroquine did not induce IL-8 production (Figure 2(a)). Similar results were obtained when NF-κB activation was measured through the reporter plasmid pNiFty-Luc (Figure 2(a)). Nontransfected HEK cells did not produce IL-8 in the presence of liposomes with Mn^{2+}-induced nonbilayer phospholipid arrangements (data not shown).

The production of IL-8 by HEK-TLR-4/MD2/CD14 or HEK-TLR-2/TLR-6 cells in response to liposomes with Mn^{2+}-induced nonbilayer phospholipid arrangements was dose-dependent, and the effect was inhibited by chloroquine (Figure 2(b)). Cell viability in the presence of chloroquine was 90% or higher, and chloroquine did not induce apoptosis of these cells at the tested concentrations (see Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/369462). Thus, the effects observed in the presence of chloroquine can be attributed to a reversion of Mn^{2+}-induced nonbilayer phospholipid arrangements by this drug.

Additionally, we found that nonbilayer phospholipid arrangements induce the production of the proinflammatory cytokine TNF-α by BMDM from BALB/c mice. The production of TNF-α induced by smooth liposomes was significantly lower. Furthermore, anti-TLR-2 and anti-TLR-4 antibodies blocked the production of TNF-α by BMDM in response to nonbilayer phospholipid arrangements (Figures 2(c)-2(d)).

3.3. Proinflammatory Cytokines Are Found in the Sera of Mice with a Disease Resembling Human Lupus. Liposomes with nonbilayer phospholipid arrangements induced by Mn^{2+} or chlorpromazine were used to produce an autoimmune disease resembling human lupus in mice. Antibodies against nonbilayer phospholipid arrangements were detected 1 month after the first injection of liposomes with nonbilayer phospholipid arrangements, and the titers in mice injected with chlorpromazine-induced nonbilayer phospholipid arrangements were higher than in those injected with Mn^{2+}-induced nonbilayer phospholipid arrangements ($p < 0.001$). These antibodies appeared 1 month before the anticardiolipin, anti-histone, and anti-coagulant antibodies (Figures 3(a), 3(b), 3(c), and 3(d)). The presence of the four autoantibodies confirmed that the disease had been developed in the mice. Control mice injected with TS buffer or with liposomes without nonbilayer phospholipid arrangements did not generate any of the four autoantibodies.

IL-6, IL-10, IL-12p70, IFN-γ, TNF-α, and MCP-1 were found in the sera of mice injected with liposomes with Mn^{2+}-induced nonbilayer phospholipid arrangements; IL-6, IFN-γ, and TNF-α appeared 1 month after treatment, while IL-10, IL-12p70, and MCP-1 were found after 2 months. These cytokines were also found in the sera of mice injected with chlorpromazine-induced nonbilayer phospholipid arrangements; IL-6, TNF-α, and MCP-1 appeared 1 month after treatment, while IFN-γ and IL-12p70 were found 4 months after treatment. None of the tested cytokines were found in the sera of mice treated with smooth liposomes (Figure 4).

3.4. C3, C5, TLR-4, and TLR-4-Signaling Molecules and IFN-β Are Overexpressed in the Spleens of Mice with an Autoimmune Disease Resembling Human Lupus. We evaluated gene expression in the spleens of mice from the four treatment groups: group 1, mice injected with TS buffer alone (Control I); group 2, mice injected with smooth liposomes (liposomes without nonbilayer phospholipid arrangements, Control II); group 3, mice that received liposomes with Mn^{2+}-induced nonbilayer phospholipid arrangements (Mn group); and group 4, mice that received liposomes with

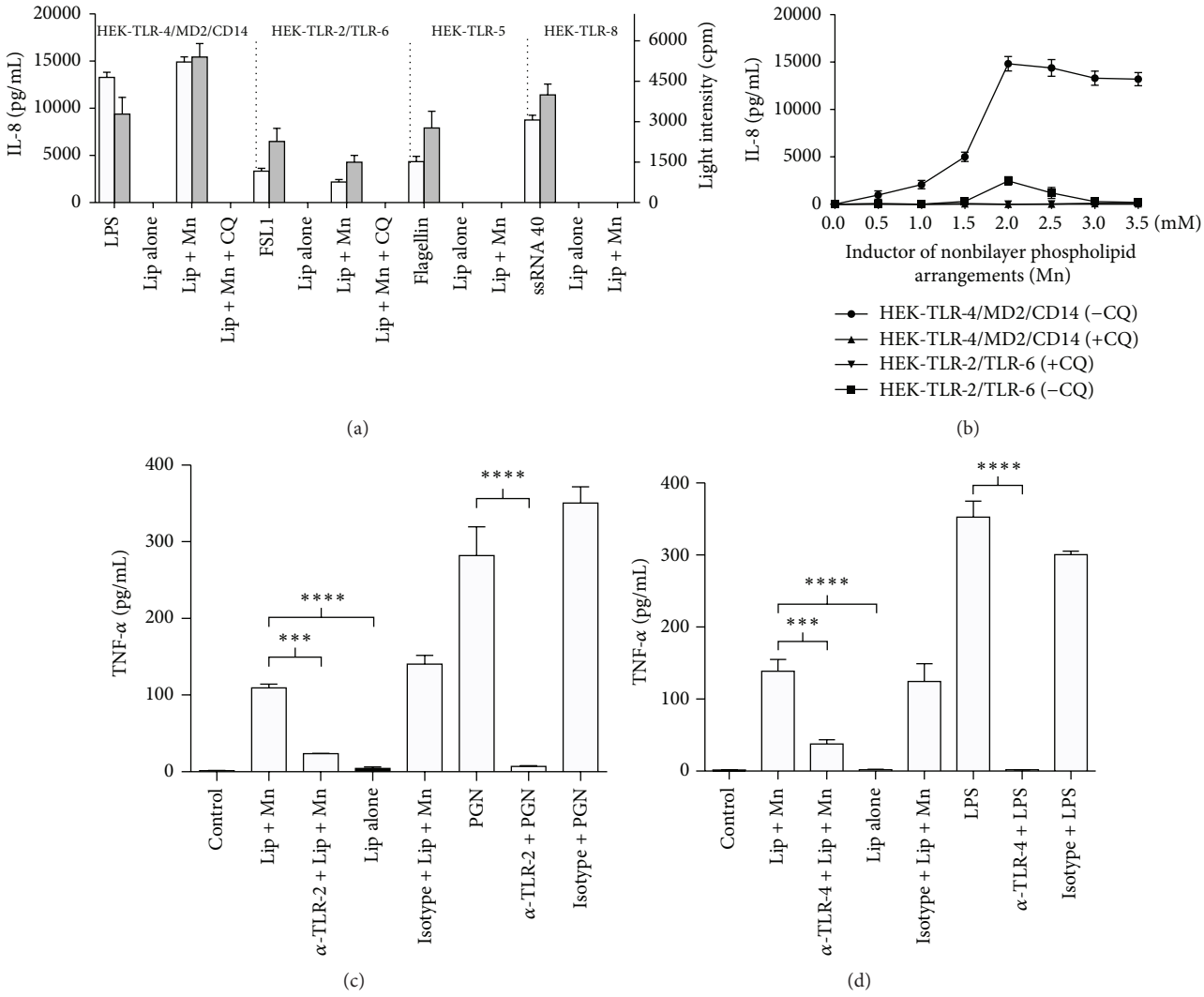

Figure 2: Liposomes with nonbilayer phospholipid arrangements induce TLR-2/6 and TLR-4 signaling in TLR-expressing HEK cells. (a) HEK-TLR-4/MD2/CD14, HEK-TLR-2/TLR-6, HEK-TLR-5, and HEK-TLR-8 cells were stimulated for 24 h at 37°C with 10 μL of egg-yolk phosphatidylcholine/egg-yolk phosphatidic acid (2 : 1) liposomes (50 nmol phosphatidic acid in 50 μL TS buffer) alone or bearing nonbilayer phospholipid arrangements induced with 2 mM $MnCl_2$. As positive controls, cells were stimulated with their TLR agonist: 100 ng/mL LPS, 1 μg/mL FSL1, 1 μg/mL bacterial flagellin, or 2.5 μg/mL ssRNA40, respectively. As negative controls, cells were stimulated with liposomes alone or bearing nonbilayer phospholipid arrangements + 0.1 mM chloroquine (CQ). IL-8 levels and NF-κB activation were measured 24 h after stimulation. (b) HEK-TLR-4/MD2/CD14 and HEK-TLR-2/TLR-6 cells were stimulated with nonbilayer phospholipid arrangements induced with different $MnCl_2$ concentrations with (+CQ) or (−CQ) 0.1 mM chloroquine. IL-8 levels were measured 24 h after stimulation. Histograms and graphs represent the mean ± SD of three independent experiments. (c, d) TLR-2 and TLR-4 of bone marrow-derived macrophages (BMDM) were analyzed. (c) 4×10^4 BMDM were stimulated with liposomes alone or bearing Mn^{2+}-induced nonbilayer phospholipid arrangements (as in a). Where indicated, 10 μg/mL of anti-TLR-2 or isotype control (IgG1,κ) was added 2 h before liposomes addition. PGN (1 μg/mL) was the positive control. After 24 h at 37°C, TNF-α levels were determined. (d) 3×10^3 BMDM were stimulated with 20 μL of liposomes (as in a). Where indicated, 20 μg/mL of anti-TLR-4 or isotype control (IgG2a,κ) was added 2 h before liposomes addition. LPS (10 ng/mL) was the positive control. TNF-α levels were determined as in (c). Histograms represent the mean ± SD of three independent experiments. Data were analyzed with one-way ANOVA and Tukey's multiple comparisons test. $^{****}p \leq 0.0001$, $^{***}p \leq 0.001$.

chlorpromazine-induced nonbilayer phospholipid arrangements (CPZ group). Spleens were collected 4 months after treatment, and the cRNA derived from the spleens of three mice from each group were pooled and hybridized to a whole mouse genome microarray chip.

No significant differences were found between Control I and Control II groups; 426 genes were overexpressed and 62 genes were underexpressed in the Mn group, compared with the Control II group; 542 genes were overexpressed and 73 genes were underexpressed in the CPZ group, compared with the Control II group; and 383 genes were overexpressed and 44 genes were underexpressed in the CPZ group, compared with the Mn group. Table 1 shows a list of genes that were overexpressed in both the Mn and CPZ groups, compared

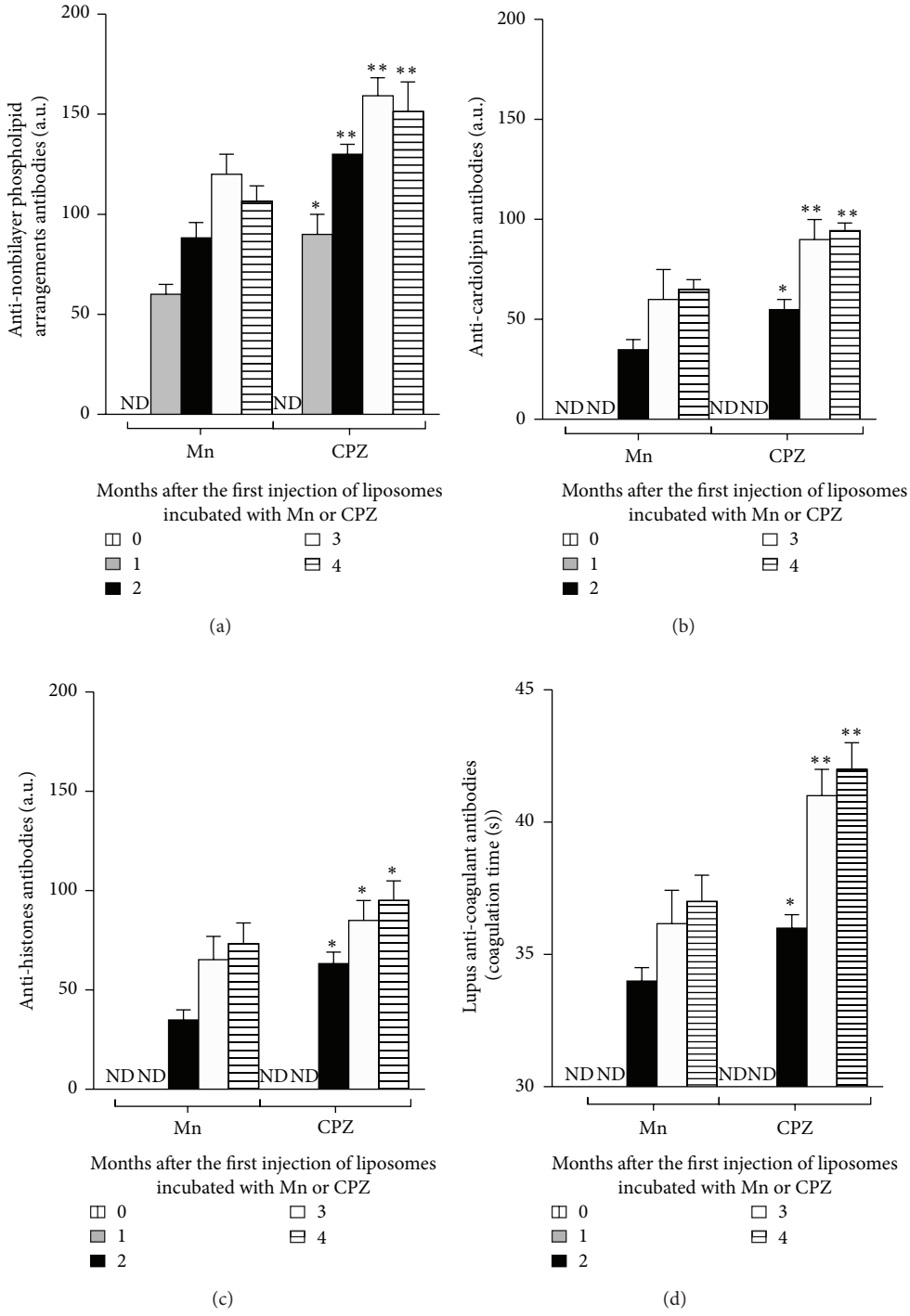

FIGURE 3: Antibodies found in the sera of mice with the disease resembling human lupus. Mice (10 per group) were injected with egg-yolk phosphatidylcholine/egg-yolk phosphatidic acid (2 : 1) liposomes (50 nmol phosphatidic acid in 50 μL TS buffer), alone (negative control) or incubated 30 min at 37°C with 5 mM $MnCl_2$ or 3 mM chlorpromazine (CPZ) to induce nonbilayer phospholipid arrangements. Mice injected with TS buffer were also used as negative control. (a) Anti-nonbilayer phospholipid arrangements, (b) anti-cardiolipin (CL), (c) anti-histone, and (d) lupus anti-coagulant antibodies were measured before and 1–4 months after liposome injection. Histograms represent the mean ± SD of three independent experiments. Control mice injected with TS buffer or with liposomes without nonbilayer phospholipid arrangements did not have detectable levels of any of these four antibodies, 1 or 4 months after the administration of TS or liposomes. ND: not detected. Data were analyzed with Kruskal-Wallis test with Dunn's post-test (GraphPad Prism). $^{*}p < 0.05$; $^{**}p < 0.01$. Asterisks indicate statistical significance between the antibody titers induced by liposomes incubated with CPZ and those incubated with Mn.

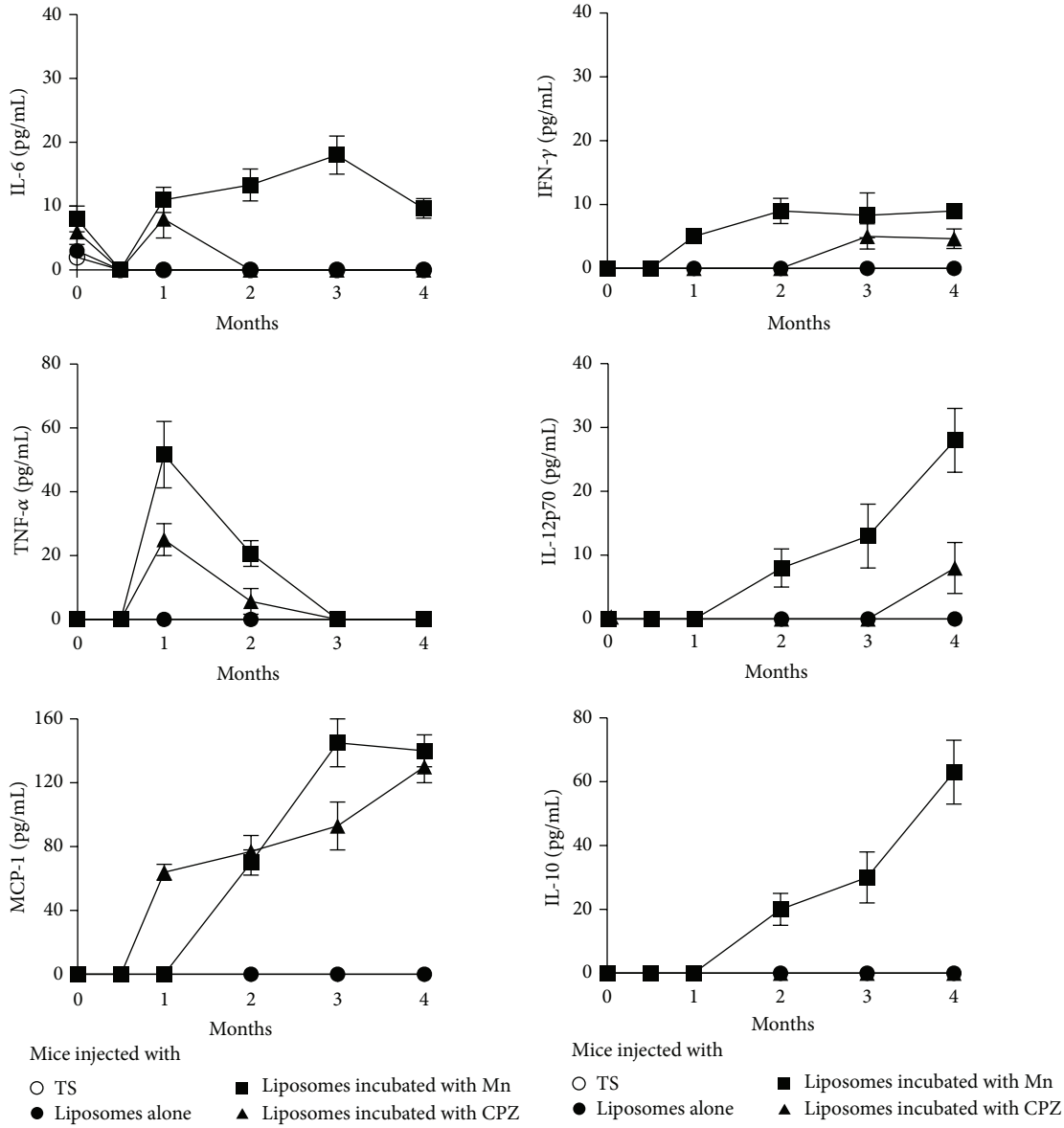

Figure 4: Proinflammatory cytokines are found in the sera of mice with the disease resembling human lupus. Cytokines were detected in the sera of mice injected with egg-yolk phosphatidylcholine/egg-yolk phosphatidic acid (2 : 1) liposomes (50 nmol phosphatidic acid in 50 μL TS buffer) incubated with 5 mM $MnCl_2$ or 3 mM chlorpromazine (CPZ). Months indicate the time after the first injection of liposomes. The detection limits for each cytokine were 5 pg/mL IL-6, 7.3 pg/mL TNF-α, 52.7 pg/mL MCP-1, 2.5 pg/mL IFN-γ, 10 pg/mL IL-12p70, and 17.5 pg/mL IL-10. Control groups (mice injected with TS buffer or with liposomes without nonbilayer phospholipid arrangements) did not have detectable levels of these cytokines. Bars represent the mean ± SD of three independent experiments with six mice each.

with the Control II group. This includes genes for complement components (C3 and C5), molecules involved in the presentation of exogenous antigens, in the production of antibodies, and in TLR-4 and NOD-2 signaling. Table 1 also shows a list of genes that were underexpressed in both the Mn and CPZ groups, compared with the Control II group. These are genes for molecules that are involved in apoptosis and in NK cell recognition.

The C3 and C5 complement proteins were increased in the Control I and Control II groups, compared with the Mn and CPZ groups. However, C3a and C5a, two active fragments that are produced by C3 and C5 cleavage, were increased in the Mn and the CPZ groups, compared with the Control I and Control II groups (Figures 5(a)-5(b)). IFN-β was also increased in the spleens of mice with the autoimmune disease, compared with healthy mice (Figure 5(c)). The number of cells expressing TLR-4 increased in the Mn and the CPZ groups, compared with the Control I and Control II groups (Figure 5(d)).

3.5. NKT, Dendritic, and B Cells Are Increased in the Spleens of Mice with an Autoimmune Disease Resembling Human Lupus.

Table 1: Gene expression in mice with a disease resembling human lupus triggered by liposomes with Mn^{2+}-induced or chlorpromazine-induced nonbilayer phospholipid arrangements.

	Genes
Pathways with overexpression of genes	
Complement system	Classical pathway: *C1ra*, *C1s*, *C1q*, *C3*, *C5*, and *C7* Receptors of the classical pathway: *C3ar1* and *C5ar1* Alternative pathway: *Cfd*, *Cfh*, and *Cfhr2*
Exogenous antigen presentation	*H2-aa*, *H-2aa*, *H2-dma*, and *Clip*
Antibody production	*Igh-vj558*, *Lyn*, *Syk*, *Plcg2*, *Can*, *Akt1*, and *Nfkb1*
TLR-4 signalling	*Tlr-4*, *Tram*, *Trif*, *Tbk1*, *Irf3*, *Ifn-α*, and *Ifn-β*
NOD-2 signalling	*Nod-2*, *Ripk2*, *Card9*, *Mapk10*, and *Tnfa*
Pathways with underexpression of genes	
Apoptosis	*Casp8*, *Cycs*, *Apaf1*, and *Aifm1*
Recognition of NK cells	*Klrb1a*, *Klrb1c*, *Klra23*, *Klra7*, *Gzmb*, and *Klra22*

Genes that were over- or underexpressed in mice injected with Mn-induced nonbilayer phospholipid arrangements (Mn group) or chlorpromazine-induced nonbilayer phospholipid arrangements (CPZ group), compared with mice injected with liposomes without nonbilayer phospholipid arrangements (Control II). The cutoff for over- and underexpressed genes was set as mean fold change $\log_2$ ratio greater than +2 or lower than −2, as assessed by two-way ANOVA, with $p < 0.01$.

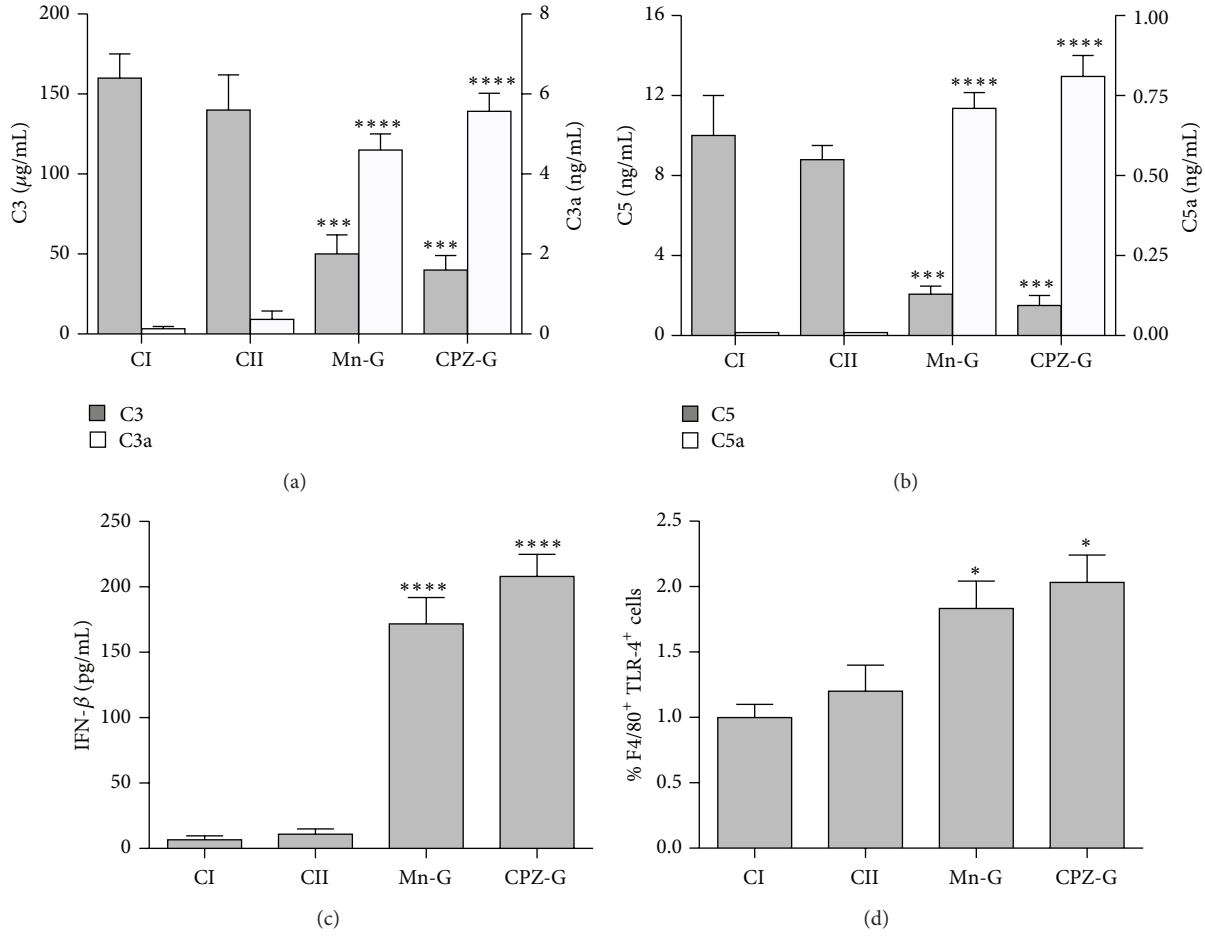

Figure 5: C3, C5, TLR-4, and IFN-β are overexpressed in the spleens of mice with a disease resembling human lupus. The complement proteins C3 and C5 (a), the fragments C3a and C5a (b) (produced by C3 and C5 cleavage), and IFN-β (c) were detected in a cell-free extract from the spleen of mice injected with TS buffer, Control I group (CI), egg-yolk phosphatidylcholine/egg-yolk phosphatidic acid (50 nmol phosphatidic acid in 50 μL TS) liposomes alone, Control II group (CII), or bearing Mn^{2+}-induced nonbilayer phospholipid arrangements (Mn-G) or chlorpromazine-induced nonbilayer phospholipid arrangements (CPZ-G). The detection ranges were C3 (4.69–300 μg/mL), C3a (0.16–10 ng/mL), C5 (0.25–16 ng/mL), C5a (0.062–4.0 ng/mL), and IFN-β (7.8–500 pg/mL). TLR-4 (d) was detected by flow cytometry on cells obtained from fresh spleens from the four treatment groups. Bars represent the mean ± SD of three independent experiments, with three mice in each of the four groups. Data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. $^{****}p \leq 0.0001$, $^{***}p \leq 0.001$, and $^{*}p \leq 0.05$. Asterisks indicate statistical significance between the control groups and the Mn or CPZ groups.

Activated CD4 and CD8 T cells (Figures 6(a), 6(b), 6(c), and 6(d)), NKT cells (Figures 6(e)-6(f)), activated dendritic cells (Figures 6(g)-6(h)), and activated and TLR4 expressing B1 and B2 cells (Figures 6(i), 6(j), and 6(k)) were identified by flow cytometry in the spleens of mice. Fifteen days after the mice were injected intrasplenically with liposomes bearing nonbilayer phospholipids arrangements induced by Mn or chlorpromazine, the percentage and activation of CD4 and CD8 T cells were not increased, compared with the control mice that received TS buffer or liposomes alone (Figures 6(l)-6(m)). In contrast, the percentage of NKT, dendritic, and B2 cells was increased (Figures 6(n), 6(o), and 6(q)), and the activation of dendritic and B2 cells was also increased (Figures 6(o)-6(q)). An increase in TLR4 expression was also observed in B2 cells (Figure 6(q)). B1 cells did not increase in percentage, but the number of activated and TLR4 expressing B1 cells did increase (Figure 6(p)).

4. Discussion

SLE is a systemic autoimmune disease of unknown etiology characterized by B and T cell hyperactivity, by defects in the clearance of apoptotic cells and immune complexes, and by production of a complex mixture of various cytokines, chemokines, signaling molecules, and pattern-recognition receptors involved in immunity [4, 24]. We have previously demonstrated that liposomes with nonbilayer phospholipid arrangements trigger a disease that resembles human lupus in mice and that IgM and IgG specific to nonbilayer phospholipid arrangements are produced in these mice. Now, we demonstrate that nonbilayer phospholipid arrangements are agonists for TLR-4/MD-2. The activation of this innate immune receptor leads to the production of proinflammatory cytokines; a proinflammatory environment is needed for efficient activation of the adaptive immune response and the production of IgG antibodies. These findings were supported by the increase in the percentage of NKT cells and by the increase in the percentage and activation of dendritic and B2 cells. In addition, the activation of TLR-4/MD2/CD14 by liposomes with Mn^{2+}-induced nonbilayer phospholipid arrangements supports our hypothesis on the similarity of the structure of conical phospholipids, which form an inverted micelle inside the nonbilayer arrangement, with the conical association of the acyl chains of the lipid A moiety of LPS.

The importance of the lipid A moiety of LPS was taken into account in the design of glucopyranosyl lipid A (GLA), a synthetic lipid A with six acyl chains and a single phosphate group. GLA as a stable oil-in-water-emulsion (GLA-SE) is a TLR-4 agonist, which signals through MyD88 and TRIF and drives a polyclonal T_H1 response *in vivo*, characterized by IFN-γ, TNF-α, and IL-2 producing cells and IgG2c isotype switching [25, 26].

We performed our TLR activation assays in HEK cells transfected with various human TLRs. Interestingly, we also found that nonbilayer phospholipid arrangements induce the production of the proinflammatory cytokine TNF-α by BALB/c mouse BMDM. Furthermore, anti-TLR-2 and anti-TLR-4 antibodies blocked the production of TNF-α by these macrophages in response to nonbilayer phospholipid arrangements. These findings confirmed our observations with the HEK cells transfected with human TLRs, which also showed that nonbilayer phospholipid arrangements are agonists for TLR-4/MD-2 and TLR-2/TLR-6.

We observed that liposomes with nonbilayer phospholipid arrangements were agonists for TLR-2/TLR-6, but the activation was 3-fold lower than for TLR-4/MD2/CD14. Bacterial macroamphiphilic molecules, such as lipoproteins (including the synthetic lipoprotein FSL-1), lipoteichoic acids, lipoglycans, glycolipids, and lipoarabinomannans, are anchored on bacterial envelopes through a lipidic structure, which is usually a diacylglyceryl moiety. These amphiphilic molecules are mainly recognized via their lipid anchor through TLR-2, alone or as a heterodimer with TLR-1 or TLR-6 [27, 28]. Because the liposomes bearing nonbilayer phospholipid arrangements are made of phosphatidylcholine and phosphatidate, which also have the diacylglyceryl moiety, it is possible that this lipid moiety activated the TLR-2/TLR-6 heterodimer.

TLRs not only recognize pathogen-associated molecular patterns, such as LPS, but also recognize damage-associated molecular patterns, which are released by cells that are either under stress or undergoing apoptosis or necrosis [29]. Examples of damage-associated molecular patterns that are TLR-4 agonists include heat-shock protein 60, fibronectin, fibrinogen, α-defensins, and hyaluronan. The molecular structure of these agonists is different from that of LPS, but they all have hydrophobic regions, which are probably recognized by TLR-4 [30]. The modification of the lipid bilayer of cell membranes could be a signal of cell stress: nonbilayer phospholipid arrangements are normally transitory, but if they are stabilized by Mn^{2+} or by the drugs chlorpromazine or procainamide, they could activate the innate immune response via TLRs and then induce the production of antibodies, with the subsequent development of an autoimmune disease.

TLR-4 signaling leads to the activation of NF-κB and the production of proinflammatory cytokines, including TNF-α, IL-12, and IFN-γ, and chemokines, such as MCP-1. We found these cytokines and chemokines in the sera of mice treated with liposomes with Mn^{2+}- or chlorpromazine-induced nonbilayer phospholipid arrangements. The increase in the concentration of the proinflammatory cytokines IL-6 and TNF-α correlated with the appearance of anti-nonbilayer phospholipid arrangement antibodies 1 month after the first injection of mice with nonbilayer phospholipid arrangements, and this also corresponds to the period of disease onset. The chemokine MCP-1 and the proinflammatory cytokines INF-γ and IL-12p70 increased between months 2 and 4 and correlated with the development and establishment of the disease, given by an increase in the titers of anti-nonbilayer phospholipid arrangement antibodies and the presence of anti-cardiolipin, anti-histone, and anti-coagulant antibodies. IL-10 was only detected in mice that received Mn^{2+}-induced nonbilayer phospholipid arrangements. The proinflammatory cytokines IL-1, IL-6, IFN-γ, and TNF-α and the immunomodulatory cytokines IL-10 and tumor growth factor-β (TGF-β) have been identified as important players

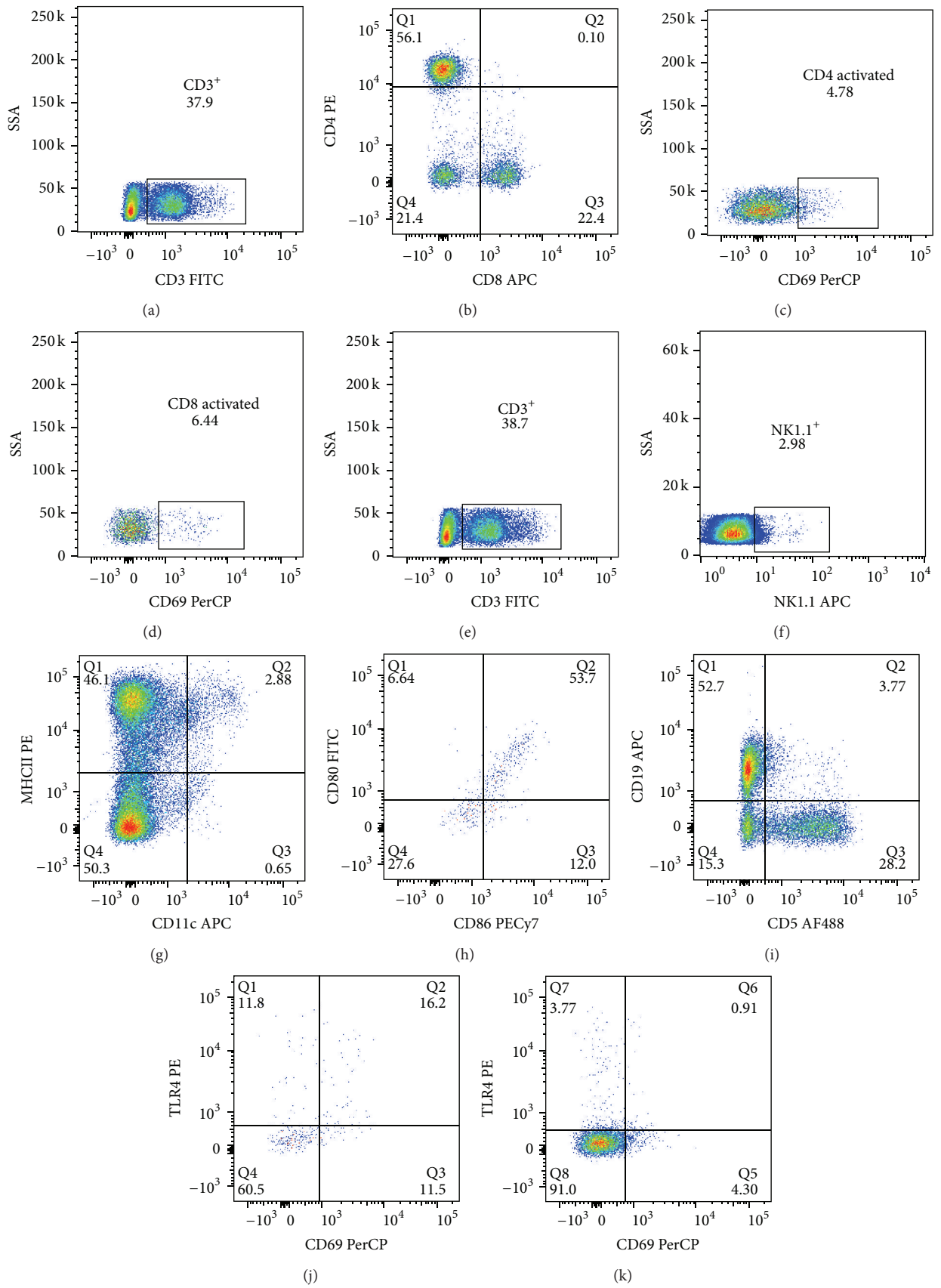

Figure 6: Continued.

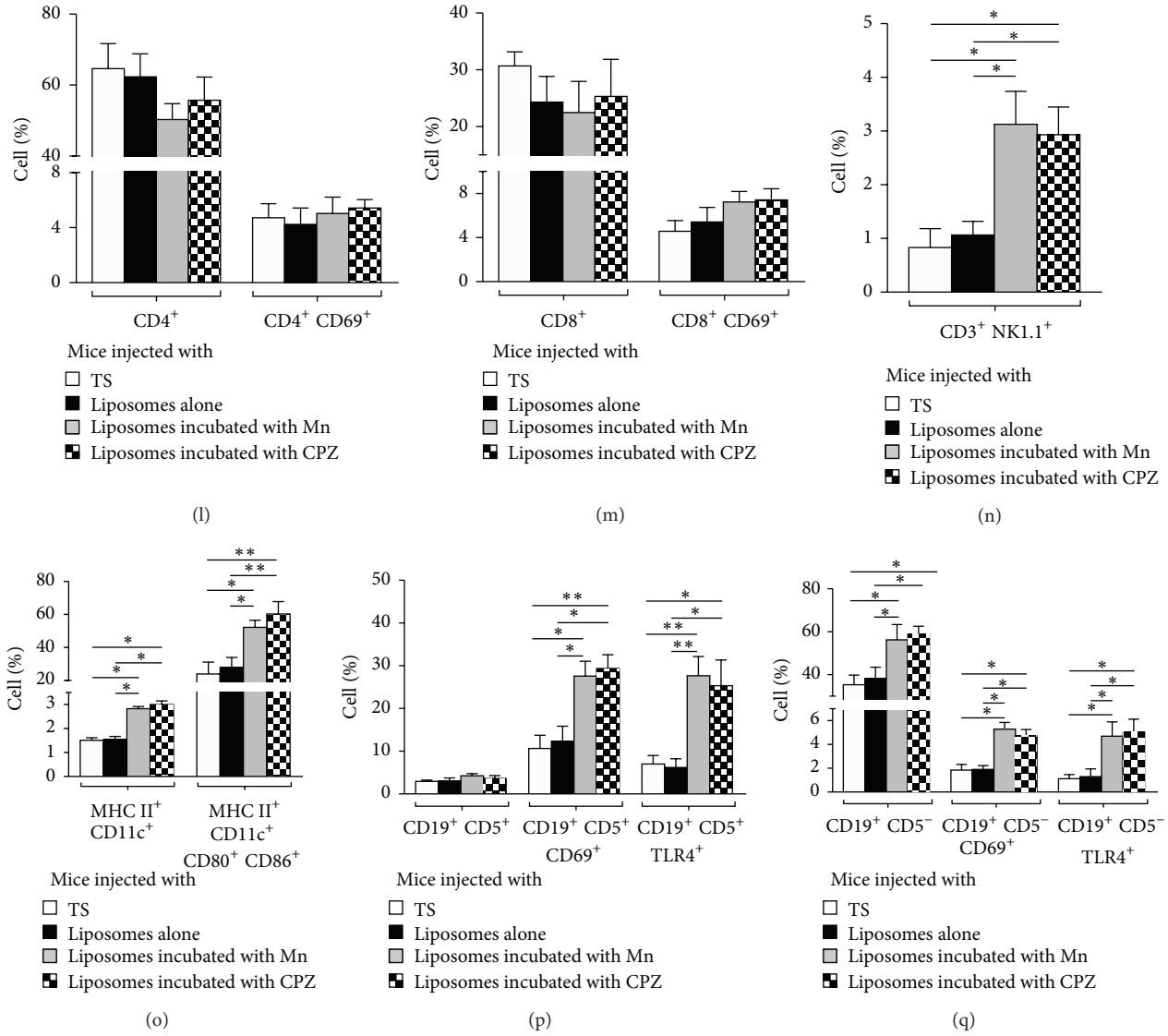

Figure 6: Dendritic, B1, and B2 cells are activated in mice with an autoimmune disease resembling human lupus. To analyze the percentage and activation of immune cells, cell suspensions from the spleens of mice injected with TS buffer, liposomes alone, or liposomes incubated with Mn or CPZ were labeled with antibodies and analyzed by flow cytometry. Gating strategy for the identification of activated CD4 ($CD3^+$, $CD4^+$, $CD8^-$, and $CD69^+$) (a–c) and CD8 ($CD3^+$, $CD4^-$, $CD8^+$, and $CD69^+$) (a, b, and d) T cells; NKT cells ($CD3^+$, $NK1.1^+$) (e, f); activated dendritic cells ($MHCII^+$, $CD11c^{++}$, $CD80^+$, and $CD86^+$) (g, h); activated B1 ($CD19^+$, $CD5^+$, and $CD69^+$) and B2 ($CD19^+$, $CD5^-$, and $CD69^+$) cells; and expression of TLR-4 (i–k). Percentage of total NKT (n); total and activated CD4 (l) and CD8 (m) T cells, dendritic cells (o), and B1 (p) and B2 (q) cells. The expression of TLR-4 was evaluated on B1 (p) and B2 (q) cells. Kruskal-Wallis test with Dunn's post-test was used for statistical analysis; significance was set at $p < 0.05$. Asterisks represent statistically significant differences between the indicated groups ($^*p < 0.05$, $^{**}p < 0.01$).

in the development of SLE [31, 32]. The cytokine pattern that we report indicates another similarity of this mouse model with the human disease.

TLR-4 was increased at the mRNA level and the number of cells that express TLR-4 increased in the spleens of mice that received liposomes with nonbilayer phospholipid arrangements. Other genes associated with TLR-4 signaling, such as *Tram*, *Trif*, *Tbk1*, and *Irf3*, were also increased at the mRNA level in these mice. These genes are associated with TRIF-dependent, but not MyD88-dependent, TLR-4 signaling [33]. TRIF-dependent TLR-4 signaling leads to the production of IFN-α and β. IFN-β was increased in the spleens of mice that had received liposomes with nonbilayer phospholipid arrangements compared with healthy mice, and increased levels of IFN-α and β are reported in patients with SLE [34].

The expression of genes associated with the classical pathway of complement activation (*C1ra*, *C1s*, *C1q*, *C3*, *C5*, and *C7*) was increased in mice that had received liposomes with nonbilayer phospholipid arrangements, and this mRNA increase correlated with the detection of C3a and C5a proteins in the spleens of mice with the autoimmune disease.

Complement has an important role in the immune response, but it also has the potential to cause tissue damage, as has been reported in SLE and other autoimmune diseases [35, 36]. It will be interesting to evaluate the role of complement in the tissue damage that is observed in this mouse model of autoimmune disease.

In contrast, the expression of genes associated with NK cell activation (*Klrb1a*, *Klrb1c*, *Klra23*, *Klra7*, *Gzmb*, and *Klra22*) was decreased in mice that received liposomes with nonbilayer phospholipid arrangements. This decrease could reflect a reduction in the absolute number of NK cells or a lower activation of the existing NK cells. The expression of genes associated with apoptosis (*Casp8*, *Cycs*, *Apaf1*, and *Aaifm1*) was also decreased in mice that received liposomes with nonbilayer phospholipid arrangements. This could be relevant for disease development, since deficient apoptosis could favor the survival of autoreactive T cells.

An important additional support for our hypothesis on the effect of nonbilayer phospholipid arrangements on the innate immune response is our finding that mice with the autoimmune disease resembling human lupus have an increase in NKT and dendritic cell percentages, together with increased dendritic cell activation. These cells could recruit and activate B1 and B2 cells, which are the precursors of plasma cells that produce antibodies against nonbilayer phospholipid arrangements.

5. Conclusions

The findings reported in this paper are consistent with a mouse model in which nonbilayer phospholipid arrangements directly activate TLR-4 and TLR-2/TLR-6 and lead to the production of proinflammatory cytokines. The proinflammatory environment leads to the efficient activation of the adaptive immune response to the production of IgG antibodies specific for nonbilayer phospholipid arrangements. These antibodies bind to the nonbilayer phospholipid arrangements that are transitorily formed on the surface of many cells and cause cell lysis; the exposure of intracellular antigens could then lead to the formation of anti-cardiolipin, anti-histone, and anti-coagulant antibodies. Furthermore, the inflammatory environment can cause complement-mediated tissue damage and IFN-β production. Thus, this mouse model of autoimmune disease recapitulates many features of human lupus.

Conflict of Interests

Carlos Wong and Isabel Baeza are listed as authors in the following patents: (1) US Patent 6,777,193, Methods for diagnostic and/or treatment of antiphospholipid antibodies-related diseases and devices, and (2) US Patent 7,867,723, Methods for antiphospholipid syndrome.

Authors' Contribution

Carlos Wong-Baeza and Isabel Baeza designed the study; Carlos Wong-Baeza, Alonso Tescucano, Albany Reséndiz, Luis España, and Jeanet Serafín-López performed experiments; Horacio Astudillo analyzed the microarray data; Carla Landa evaluated cell populations on mice; Carlos Wong-Baeza, Iris Estrada-García, Sergio Estrada-Parra, Leopoldo Flores-Romo, Carlos Wong, and Isabel Baeza analyzed and interpreted the data; Isabel Baeza wrote the paper. All authors have revised and approved the final version of the paper.

Acknowledgments

This study was supported by grants from the National Polytechnic Institute, Mexico (SIP20130949 to Isabel Baeza and SIP20131017 to Carlos Wong). The authors thank Isabel Baeza for discussion and assistance in the preparation of the English paper.

References

[1] L. Geng, X. Li, X. Feng et al., "Association of TNF-α with impaired migration capacity of mesenchymal stem cells in patients with systemic lupus erythematosus," *Journal of Immunology Research*, vol. 2014, Article ID 169082, 14 pages, 2014.

[2] D. Wang, H. Zhang, J. Liang et al., "Allogeneic mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus: 4 years of experience," *Cell Transplantation*, vol. 22, no. 12, pp. 2267–2277, 2013.

[3] J. B. Rottman and C. R. Willis, "Mouse models of systemic lupus erythematosus reveal a complex pathogenesis," *Veterinary Pathology*, vol. 47, no. 4, pp. 664–676, 2010.

[4] S.-L. Yu, W.-P. Kuan, C.-K. Wong, E. K. Li, and L.-S. Tam, "Immunopathological roles of cytokines, chemokines, signaling molecules, and pattern-recognition receptors in systemic lupus erythematosus," *Clinical & Developmental Immunology*, vol. 2012, Article ID 715190, 14 pages, 2012.

[5] C. Chang and M. E. Gershwin, "Drugs and autoimmunity—a contemporary review and mechanistic approach," *Journal of Autoimmunity*, vol. 34, no. 3, pp. J266–J275, 2010.

[6] C. D. Vedove, J. C. Simon, and G. Girolomoni, "Drug-induced lupus erythematosus with emphasis on skin manifestations and the role of anti-TNFα agents," *Journal of the German Society of Dermatology*, vol. 10, no. 12, pp. 889–897, 2012.

[7] D. Feng, L. Yang, X. Bi, R. C. Stone, P. Patel, and B. J. Barnes, "Irf5-deficient mice are protected from pristane-induced lupus via increased Th2 cytokines and altered IgG class switching," *European Journal of Immunology*, vol. 42, no. 6, pp. 1477–1487, 2012.

[8] A. Sadanaga, H. Nakashima, M. Akahoshi et al., "Protection against autoimmune nephritis in MyD88-deficient MRL/lpr mice," *Arthritis & Rheumatism*, vol. 56, no. 5, pp. 1618–1628, 2007.

[9] S. Williams, P. Stafford, and S. A. Hoffman, "Diagnosis and early detection of CNS-SLE in MRL/lpr mice using peptide microarrays," *BMC Immunology*, vol. 15, article 23, 2014.

[10] I. Baeza, E. Leyva, B. Campos et al., "Antibodies to nonbilayer phospholipid arrangements induce a murine autoimmune disease resembling human lupus," *European Journal of Immunology*, vol. 34, no. 2, pp. 576–586, 2004.

[11] J. Jouhet, "Importance of the hexagonal lipid phase in biological membrane organization," *Frontiers in Plant Science*, vol. 4, article 494, 2013.

[12] P. R. Cullis, B. de Kruijff, M. J. Hope, R. Nayar, and S. L. Schmid, "Phospholipids and membrane transport," *Canadian Journal of Biochemistry*, vol. 58, no. 10, pp. 1091–1100, 1980.

[13] D. E. Vance and J. E. Vance, *Biochemistry of Lipids, Lipoproteins and Membranes*, Elsevier, London, UK, 1991.

[14] C. Wong-Baeza, R. Hernández-Pando, A. Reséndiz et al., "Molecular organization of the non-bilayer phospholipid arrangements that induce an autoimmune disease resembling human lupus in mice," *Molecular Membrane Biology*, vol. 29, no. 2, pp. 52–67, 2012.

[15] L. Aguilar, G. Ortega-Pierres, B. Campos et al., "Phospholipid membranes form specific nonbilayer molecular arrangements that are antigenic," *The Journal of Biological Chemistry*, vol. 274, no. 36, pp. 25193–25196, 1999.

[16] A. B. Schromm, K. Brandenburg, H. Loppnow et al., "Biological activities of lipopolysaccharides are determined by the shape of their lipid A portion," *European Journal of Biochemistry*, vol. 267, no. 7, pp. 2008–2013, 2000.

[17] U. Seydel, M. Oikawa, K. Fukase, S. Kusumoto, and K. Brandenburg, "Intrinsic conformation of lipid A is responsible for agonistic and antagonistic activity," *European Journal of Biochemistry*, vol. 267, no. 10, pp. 3032–3039, 2000.

[18] R. L. Kitchens and P. A. Thompson, "Modulatory effects of sCD14 and LBP on LPS-host cell interactions," *Journal of Endotoxin Research*, vol. 11, no. 4, pp. 225–229, 2005.

[19] S. I. Miller, R. K. Ernst, and M. W. Bader, "LPS, TLR4 and infectious disease diversity," *Nature Reviews Microbiology*, vol. 3, no. 1, pp. 36–46, 2005.

[20] R. I. Freshney, *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*, Wiley-Blackwell, Hoboken, NJ, USA, 2010.

[21] A. M. H. P. van den Besselaar, J. Meeuwisse-Braun, R. Jansen-Gruter, and R. M. Bertina, "Monitoring heparin therapy by the activated partial thromboplastin time—the effect of preanalytical conditions," *Thrombosis and Haemostasis*, vol. 57, no. 2, pp. 226–231, 1987.

[22] National Research Council, Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, and National Academies Press, *Guide for the Care and Use of Laboratory Animals*, National Academies Press, Washington, DC, USA, 2011.

[23] S. Shack, "Gene expression profiling of tissues and cell lines: a dual-color microarray method," *Methods in Molecular Biology*, vol. 700, pp. 125–143, 2011.

[24] X. Zhen, J. Qiao, R. Li, L. Wang, and P. Liu, "Serologic autoimmunologic parameters in women with primary ovarian insufficiency," *BMC Immunology*, vol. 15, no. 1, article 11, 2014.

[25] M. T. Orr, M. S. Duthie, H. P. Windish et al., "MyD88 and TRIF synergistic interaction is required for TH1-cell polarization with a synthetic TLR4 agonist adjuvant," *European Journal of Immunology*, vol. 43, no. 9, pp. 2398–2408, 2013.

[26] A. Pantel, C. Cheong, D. Dandamudi et al., "A new synthetic TLR4 agonist, GLA, allows dendritic cells targeted with antigen to elicit Th1 T-cell immunity *in vivo*," *European Journal of Immunology*, vol. 42, no. 1, pp. 101–109, 2012.

[27] L. Blanc, R. Castanier, A. K. Mishra et al., "Gram-positive bacterial lipoglycans based on a glycosylated diacylglycerol lipid anchor are microbe-associated molecular patterns recognized by TLR2," *PLoS ONE*, vol. 8, no. 11, Article ID e81593, 2013.

[28] K. Farhat, S. Riekenberg, H. Heine et al., "Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling," *Journal of Leukocyte Biology*, vol. 83, no. 3, pp. 692–701, 2008.

[29] C. Richez, P. Blanco, I. Rifkin, J.-F. Moreau, and T. Schaeverbeke, "Role for toll-like receptors in autoimmune disease: the example of systemic lupus erythematosus," *Joint Bone Spine*, vol. 78, no. 2, pp. 124–130, 2011.

[30] S.-Y. Seong and P. Matzinger, "Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses," *Nature Reviews Immunology*, vol. 4, no. 6, pp. 469–478, 2004.

[31] N. Jacob and W. Stohl, "Cytokine disturbances in systemic lupus erythematosus," *Arthritis Research & Therapy*, vol. 13, no. 4, article 228, 2011.

[32] K. Ohl and K. Tenbrock, "Inflammatory cytokines in systemic lupus erythematosus," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 432595, 14 pages, 2011.

[33] K. P. Murphy, C. Janeway, P. Travers, M. Walport, A. Mowat, and C. Weaver, *Janeway's Immunobiology*, Garland Science, New York, NY, USA, 2012.

[34] V. Pascual, D. Chaussabel, and J. Banchereau, "A genomic approach to human autoimmune diseases," *Annual Review of Immunology*, vol. 28, pp. 535–571, 2010.

[35] M. J. Walport, "Complement and systemic lupus erythematosus," *Arthritis Research*, vol. 4, supplement 3, pp. S279–S293, 2002.

[36] E. Ballanti, C. Perricone, E. Greco et al., "Complement and autoimmunity," *Immunologic Research*, vol. 56, no. 2-3, pp. 477–491, 2013.