

# Autoimmune Response of IgE Antibodies to Cellular Self-Antigens in Systemic Lupus Erythematosus

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## Key Words

Antinuclear antibody · Autoantibody · Autoantigen · Cytokine · IgE antibody · Systemic lupus erythematosus

## Abstract

**Background:** Systemic lupus erythematosus (SLE) patients may exhibit high total IgE and antinuclear IgE antibodies (ANA-IgE). Here, we investigated the specificity of ANA-IgE in SLE patients and the involvement of cytokines in this immune response. **Methods:** Sera from 92 SLE patients and 68 healthy controls were evaluated for the presence of antinuclear IgE antibodies by immunoperoxidase with HEp-2,000<sup>®</sup> cells and immunoblotting with IgG-depleted sera. Total IgE, IgE specific to allergens, and serum cytokine levels were determined by ELISA. **Results:** Antinuclear IgE antibodies were detected only in SLE patients (29/92, 31.5%). High total IgE was associated with ANA-IgE ( $p < 0.0001$ ), but was not associated with IgE antibodies to allergens. In the immunoblotting, ANA-IgE reacted with nucleosomes (23/29, 79.3%), dsDNA (14/29, 48.3%), SS-A/Ro (14/29, 48.3%), SS-B/La (2/29, 18.7%), Sm (14/29, 48.3%) and RNP (18/29, 62.1%). Patients with ANA-IgE had very low serum IL-4, less IL-5 than controls ( $p < 0.05$ ), more IL-10 than seronegative patients ( $p < 0.05$ ),

and unaltered IFN- $\gamma$  levels. The IL-5/IL-10 ratio was lower in ANA-IgE seropositive patients in comparison with either seronegative patients ( $p < 0.05$ ) or healthy controls ( $p = 0.001$ ). Controls displayed higher IL-5/IFN- $\gamma$  ratios than either SLE patients with ANA-IgE ( $p < 0.05$ ) or patients without these immunoglobulins ( $p < 0.01$ ). **Conclusions:** We conclude that IgE antibodies against cell autoantigens involved in protein expression, cellular proliferation, and cell death are present in patients with SLE. Interleukin-10 seems to down-regulate this IgE autoimmune response in SLE.

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## Introduction

Systemic lupus erythematosus is characterized by a loss of immune tolerance to self-antigens, intense and diversified production of autoantibodies, and multiple clinical manifestations that may vary in different ethnic groups [1–3]. Immunoglobulin E is an antibody isotype that has been mainly associated with allergies and parasitic diseases. However, some studies have shown that non-allergic SLE patients may exhibit high total IgE, and this appears to be associated with lupus activity, since al-

lergies are no more prevalent in SLE patients than in the general population [4–7]. Furthermore, the participation of IgE in lupus nephritis has been suggested by its presence in glomerular immune complex deposits in kidney biopsies from SLE patients [8], and more recently in a mouse model of systemic autoimmune disease [9].

In a previous study, we demonstrated the presence of antinuclear IgE autoantibodies in Brazilian SLE patients without allergies. However, the autoantigens recognized by these immunoglobulins were not identified on that occasion [7]. In this work, we used cellular autoantigens that are frequently recognized by antinuclear IgG autoantibodies to elucidate the specificity of serum antinuclear IgE antibodies in a Brazilian population of SLE patients, and also evaluated the levels of IL-4, IL-5, IL-10, and IFN- $\gamma$  in patients that were either seropositive or seronegative for these immunoglobulins.

## Materials and Methods

### *Patients and Control Participants*

Ninety-two Brazilian SLE patients (87 women and 5 men, median age 39 years old, range 14–73 years) living in the state of Bahia who met at least 4 criteria from the SLE classification of the American Rheumatology College [10] were included in the study. In these individuals, the duration of SLE ranged from 2 months to 24 years. Most patients (70/92, 76.1%) used prednisone, which was prescribed alone (22/92, 23.9%) or in combination with chloroquine (17/92, 18.5%), azathioprine (12/92, 13.0%), or cyclophosphamide (7/92, 7.6%). The combination of this glucocorticoid with other drugs, such as methotrexate or cyclosporine, was infrequent. Ten patients only used chloroquine, and 6 used no medication. No clinical history of allergy was documented in their medical files, and parasitic infestations were avoided by the regular use of ivermectin in patients undergoing steroid therapy. The control group in this study was 68 healthy blood donors (22 men and 46 women, median age 32 years, range 18–61 years). Consent was obtained from all individuals, and the study was approved by the ethical committee on human research of the Santa Izabel Hospital.

### *Detection of Antinuclear IgG Antibodies*

Antinuclear IgG antibodies were probed by the indirect enzyme-linked immunosorbent assay (Quanta Lite™ ANA ELISA; Inova Diagnostics, San Francisco, Calif., USA) and the indirect fluorescent antibody test with HEp-2,000® cells (Immuno Concepts, Sacramento, Calif., USA).

### *Detection of Antinuclear IgE Antibodies*

Sera were depleted of IgG by mixing 500  $\mu$ l of each sample with 500  $\mu$ l of a 50% suspension of Protein G-Sepharose beads (Zymed® Laboratories, San Francisco, Calif., USA) for 15 min with gentle agitation. After low speed centrifugation (5 min), the serum supernatants depleted of IgG were diluted 1:3 with PBS (final serum

dilution 1:6) and incubated with fixed HEp-2,000® cells for 18 h at 18–22°C. After washing with PBS (5–10 min), the cells were incubated with 25  $\mu$ l of mouse monoclonal IgG anti-human IgE (Clone G-1; Sigma, St. Louis, Mo., USA) diluted 1:100 in PBS for 3 h at 18–22°C. Following another wash with PBS, the cells were incubated with 25  $\mu$ l of goat anti-mouse IgG peroxidase conjugate (Sigma) diluted 1:100 in PBS for 30 min (18–22°C). After another wash in PBS (5–10 min), the slides were rinsed with deionized water and incubated for 30 min with activated 4-chloro-1-naphthol substrate. A rapid rinse with both PBS and water was performed, and the slides were mounted with a semi-permanent glycerol medium (pH 9.1  $\pm$  0.2) and covered with coverslips. A positive immune reaction was demonstrated by a purple-blue stained nucleus, and this was recorded with a standard light microscope at  $\times$ 400 magnification. The patterns of nuclear reactivity were recorded using, as a reference, positive IgG reactions of control sera in a Colorzyme™ ANA test (Immuno Concepts). Sera from healthy individuals were used as negative controls for ANA-IgE.

### *Antigen Specificity of ANA*

The antigen specificity of antinuclear IgG and IgE antibodies was investigated by line immunoblotting (Nucleo-9-Line; Orgentec, Marburg, Germany), which was performed using nitrocellulose strips impregnated with the following nuclear antigens: dsDNA, nucleosome, SS-A/Ro, SS-B/La, Sm, RNP/Sm, Scl-70, Jo-1 and CENP-B. Antinuclear IgG antibodies were probed according to the manufacturer's protocol. To test the specificity of ANA-IgE, sera depleted of IgG were diluted 1:6 with Nucleo-9-Line sample buffer and incubated with the nitrocellulose strips (18 h, 18–22°C). The strips were then washed 3 times with Nucleo-9-Line Tris-buffered saline and incubated with 1 ml of mouse monoclonal IgG anti-human IgE (Clone G-1; Sigma) diluted 1:1,000 in the Nucleo-9-Line sample buffer (3 h, at 37°C). The strips were washed 5 times and then incubated with 1 ml of goat IgG anti-mouse IgG biotin conjugate (Sigma) diluted 1:5,000 (1 h, 18–22°C). Following another cycle of 3 washes, the strips were incubated with 1 ml of alkaline phosphatase-avidin conjugate (Sigma) diluted 1:40,000 (15 min, 18–22°C). After 3 washes, the immunoblots were revealed with Nucleo-9-Line BCIP/NBT substrate (30 min, 18–22°C). The controls were IgG-depleted sera from healthy individuals, mouse monoclonal IgG anti-human IgE diluted 1:1,000, and alkaline phosphatase-avidin conjugate diluted 1:40,000. The immunoblots were recorded by visual inspection using a stained standard with varied patterns of reactivity.

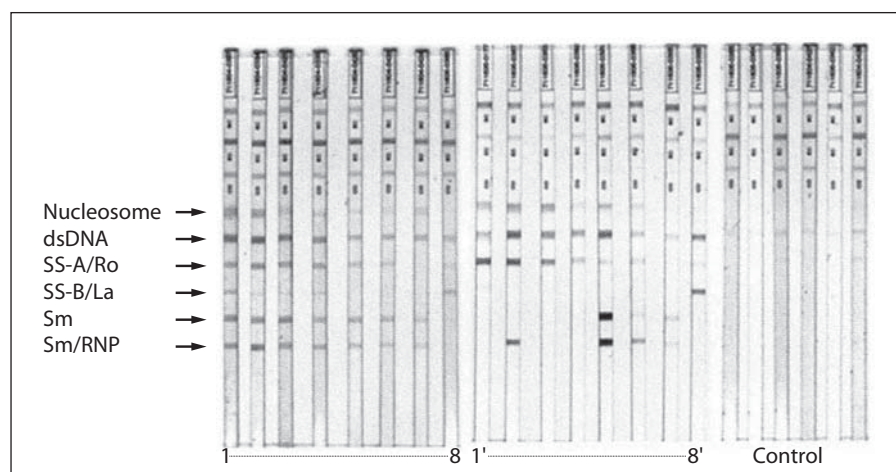
### *Measurement of Total IgE and Anti-Allergen IgE Antibodies*

Total IgE was determined by an automated immuno-enzymetric assay using the immuno-analyzer Access II (Beckman-Coulter, Calif., USA) with a working range of 0.25–3,000 IU/ml. The International Reference Value of 150 IU/ml of IgE was used in this test. Anti-allergen IgE antibodies were investigated with a capture ELISA using a house dust panel containing allergens from cockroach, mites, moulds and epithelia (Adaltis Italia, Bologna, Italy).

### *Serum Cytokine Levels*

Serum levels of IL-4, IL-5, IL-10 and IFN- $\gamma$  were determined by capture immunoassays prepared with ELISA Sets (eBioscience, San Diego, Calif., USA). These immune mediators were measured in the sera from 20 SLE women that had IgE antinuclear antibodies.

**Fig. 1.** Antigen specificity of antinuclear IgE antibodies (ANA-IgE, 1–8) and antinuclear IgG antibodies (ANA-IgG, 1'–8') in the sera from 8 SLE patients. Controls are anti-human IgE MoAb, anti-mouse IgG biotin conjugate, avidin-alkaline phosphatase conjugate, and sera from 2 healthy individuals. Antibodies were investigated using nitrocellulose strips impregnated with nuclear antigens, which are identified on the left-hand side.



ies and the sera from 20 SLE women lacking these immunoglobulins. Sera from 10 healthy women were used as controls.

#### Statistical Analyses

Continuous variables were expressed as the median and interquartile range (IQR), in accordance with their distribution in the D'Agostino-Pearson 'omnibus K2' normality test. The Kruskal-Wallis test was used to compare the medians of 3 groups, whereas the difference between the medians of 2 groups was analyzed by the Mann-Whitney U test. Fisher's exact test was used to evaluate the association between 2 categorical groups. Statistical significance was set at  $p < 0.05$ . The statistical software GraphPad Prism 5.0 was used for all statistical analyses.

## Results

### Antinuclear Antibodies

Eighty-six out of 92 (93.5%) SLE patients had detectable IgG autoantibodies, while antinuclear IgE antibodies were found in 29 of 92 SLE patients (31.5%). Three patterns of nuclear reactivity were observed with ANA-IgE: speckled (14/29, 48.3%), homogeneous (12/29, 41.4%) and nucleolar (2/29, 6.9%). The sensitivity of the ANA-IgE test was 59.3%, while its specificity was 100%. The prevalence and specificity of IgE and IgG antibodies in these 29 ANA-IgE-positive sera to purified nuclear antigens are presented in table 1 and illustrated in the immunoblots (fig. 1).

### Total and Specific IgE

The median total IgE in the SLE patient group (59.5 IU/ml, IQR = 265.8 IU/ml) was lower than that of the control group (216.5 IU/ml, IQR = 457.1 IU/ml) ( $p < 0.001$ ). Sixty SLE patients exhibited normal total IgE (22.5 IU/

**Table 1.** Antigen specificity of antinuclear IgE and IgG antibodies in SLE patients

| Nuclear antigen | ANA-IgE, n (%) | ANA-IgG, n (%) |
|-----------------|----------------|----------------|
| SSA-Ro          | 14 (48.3)      | 10 (34.5)      |
| SSB-La          | 2 (6.9)        | 2 (6.9)        |
| Sm              | 14 (48.3)      | 8 (27.6)       |
| RNP/Sm          | 18 (62.1)      | 12 (41.4)      |
| dsDNA           | 14 (48.3)      | 10 (34.5)      |
| Nucleosome      | 23 (79.3)      | 21 (72.4)      |

Antinuclear antibodies were investigated by immunoblotting in 29 SLE patients that were seropositive for ANA-IgE.

ml, IQR = 50.3 IU/ml), while 32 had increased levels of this immunoglobulin (400.5 IU/ml, IQR = 400.3 IU/ml). Nineteen out of 32 (59.4%) SLE patients with high total IgE (512 IU/ml, IQR = 437 IU/ml) were positive for ANA-IgE, while 10 out of 60 (16.7%) patients with a normal level of this immunoglobulin (78 IU/ml, IQR = 43 IU/ml) also possessed these autoantibodies. Nine out of 29 (31.0%) SLE subjects that were ANA-IgE seropositive demonstrated IgE antibodies to aeroallergens: 1 had a low level of IgE antibodies (0.68 KU/l), 5 had moderate levels of these immunoglobulins (range 1.59–2.65 KU/l), and 1 had high and 2 had very high levels (range 13.2–47.3 KU/l).

A strong association between high total IgE and the presence of antinuclear IgE antibodies was observed (Fisher's exact test,  $p < 0.0001$ ). Antinuclear IgE antibodies were not found in the healthy controls.

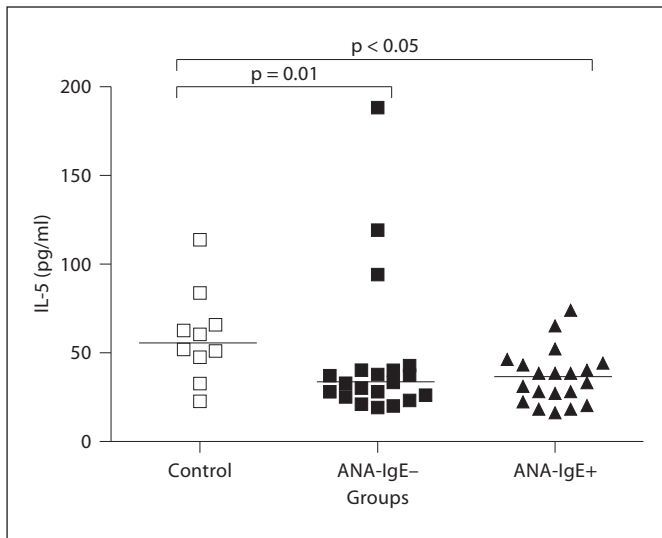


**Fig. 2.** Serum levels of IL-5 in healthy individuals (control), SLE patients that were seronegative for ANA-IgE antibodies (ANA-IgE-), and SLE subjects seropositive for these immunoglobulins (ANA-IgE+).

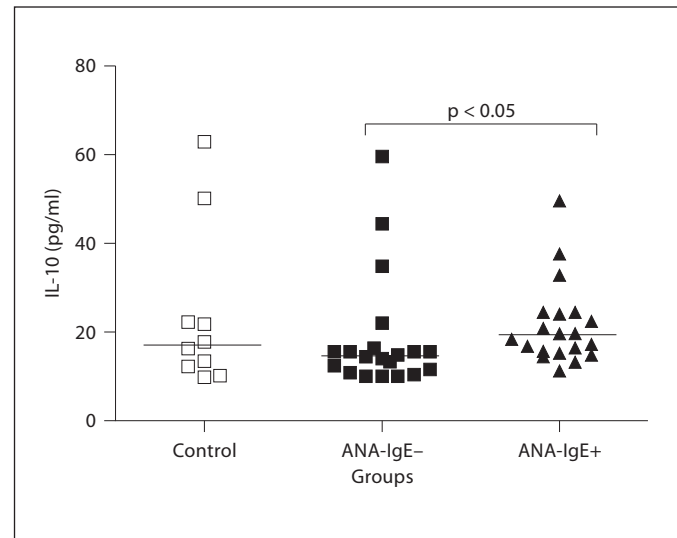


**Fig. 3.** Serum levels of IL-10 in healthy individuals (control), SLE patients that were seronegative for ANA-IgE antibodies (ANA-IgE-), and SLE subjects seropositive for these immunoglobulins (ANA-IgE+).

**Table 2.** Serum cytokine levels in healthy controls and SLE patients

| Cytokine             | Controls<br>(n = 10) | ANA-IgE-<br>(n = 20) | ANA-IgE+<br>(n = 20) | p value |
|----------------------|----------------------|----------------------|----------------------|---------|
| IL-4                 | 3.8 (4.8)            | 3.9 (5.1)            | 6.3 (2.7)            | n.s.    |
| IL-5                 | 55.1 (26.9)          | 32.9 (15.2)          | 35.2 (20.0)          | <0.05   |
| IL-10                | 16.7 (17.6)          | 14.4 (5.9)           | 18.9 (8.9)           | n.s.    |
| IFN- $\gamma$        | 29.9 (14.9)          | 29.0 (12.2)          | 27.1 (14.7)          | n.s.    |
| IL-5/IL-10           | 3.1 (1.3)            | 2.4 (1.0)            | 1.8 (0.9)            | <0.001  |
| IL-5/IFN- $\gamma$   | 1.8 (1.1)            | 1.1 (0.9)            | 1.2 (1.0)            | <0.05   |
| IFN- $\gamma$ /IL-10 | 1.7 (1.1)            | 2.3 (2.2)            | 1.6 (1.0)            | n.s.    |

Cytokine levels and cytokine ratios are expressed as medians with IQRs in parentheses. n.s. = Not significant.

Significance level according to the Kruskal-Wallis test.

### Cytokine Levels

Interleukin-4 above the analytical sensitivity of the immunoassay (2.0 pg/ml) was found in only 3 out of 20 ANA-IgE seropositive patients, in 5 out of 20 patients that were seronegative for these autoantibodies, and in 9 out of 10 controls ( $p > 0.05$ ). The highest level of IL-5 was verified in the control group in comparison with the SLE groups that were either seronegative or seropositive for

ANA-IgE ( $p < 0.05$ ). Patients with ANA-IgE displayed higher IL-10 levels than patients without these autoantibodies ( $p < 0.05$ ), but no difference was observed between the seropositive group and the control group ( $p > 0.05$ ). The levels of IFN- $\gamma$  were similar among the 3 groups evaluated ( $p > 0.05$ ; table 2, fig. 2, 3).

The IL-5/IL-10 ratio was lower in SLE patients that were ANA-IgE seropositive than in either the healthy controls or the SLE patients without these antibodies ( $p < 0.05$ ). The controls had the highest IL-5/IFN- $\gamma$  ratio when compared with either SLE patients with positive ANA-IgE ( $p < 0.01$ ) or patients without these immunoglobulins ( $p < 0.05$ ). The 3 groups exhibited similar IFN- $\gamma$ /IL-10 ratios ( $p > 0.05$ ). These results are shown in table 2 and figures 4 and 5.

### Discussion

In the present work, we identified the specificity of antinuclear IgE autoantibodies in SLE patients using IgG-depleted sera and self-antigens routinely used in rheumatology laboratories. We verified that antinuclear IgE antibodies can produce the same nuclear pattern of immune reactivity observed with antinuclear IgG antibodies in HEp-2000® cells. They react strongly in line immunoblotting with key cellular autoantigens (nucleo-

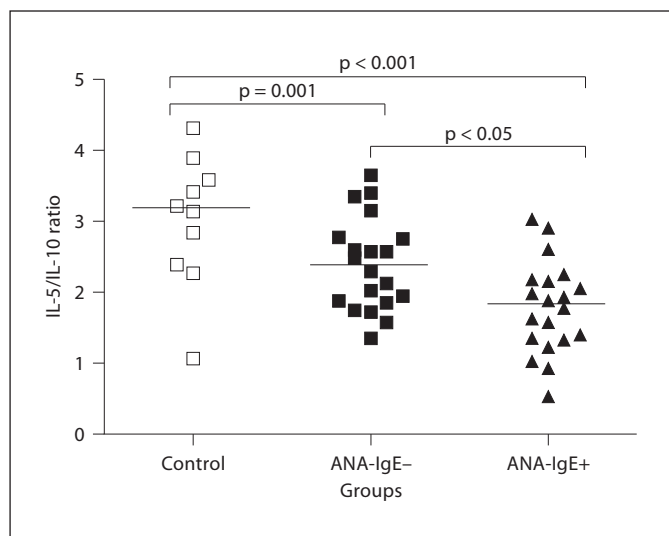


**Fig. 4.** IL-5/IL-10 ratio in healthy individuals (control), SLE patients that were seronegative for ANA-IgE antibodies (ANA-IgE-), and SLE subjects seropositive for these immunoglobulins (ANA-IgE+).

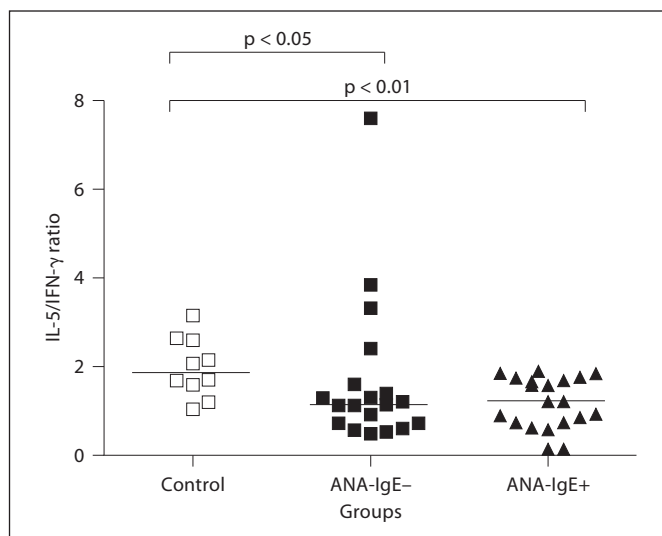


**Fig. 5.** IL-5/IFN- $\gamma$  ratios in healthy individuals (control), SLE patients that were seronegative for ANA-IgE antibodies (ANA-IgE-), and SLE subjects seropositive for these immunoglobulins (ANA-IgE+).

somes, SS-A/Ro and dsDNA) whose autoantibodies have been correlated with important clinical manifestations of SLE or are used as criteria for SLE classification (Sm and dsDNA antibodies) [11–17].

Interestingly, sera from some SLE patients reacted more intensely or identified additional nuclear antigens in IgE immunoblotting compared to IgG immunoblotting. Furthermore, the autoantigens dsDNA, Sm and RNP were more frequently recognized by IgE antibodies than by IgG antibodies in sera that were reactive for ANA-IgE, suggesting that this autoantibody isotype may improve the diagnosis of SLE in individuals exhibiting clinical manifestations of this disease but without demonstrable IgG autoantibodies. Although a strong association was verified between high total IgE and the presence of antinuclear IgE antibodies, no false-positive results were observed in the control sera, which exhibited more total IgE than did SLE sera. Moreover, the prevalence of ANA-IgE was not affected by the presence of IgE antibodies to aeroallergens. Together, these findings provide evidence that positive ANA-IgE tests in SLE patients are not false-positive results caused either by high total IgE levels or the immune cross-reactivity of anti-allergen IgE antibodies.

The cytokine profile of ANA-IgE-positive patients was characterized by low IL-4 and IL-5, unaltered levels of IFN- $\gamma$ , and elevated levels of IL-10. IL-4 and -5 are

important Th2 immune mediators of IgE-allergic inflammation, the involvement of which in the immunopathogenesis of SLE has not been documented; whereas IFN- $\gamma$  is a Th1 cytokine that polarizes the Th2 immune response, an increase or decrease of which may contribute to SLE immunopathogenesis at different stages. In contrast, IL-10 induces dsDNA autoantibodies and inhibits the activation of antigen-presenting cells. Although the immune response of IgE antibodies to nuclear antigens was not correlated with arthritis, photosensitivity or antiphospholipid syndrome, its association with increased serum levels of IL-10 suggests its relationship with lupus activity [18–20].

Our findings of elevated IL-10 and low IL-5/IL-10 ratios in ANA-IgE-seropositive patients with a normal prevalence of allergen IgE antibodies and no clinical evidence of allergy suggest that this cytokine may down-regulate allergic inflammation in this disease. Such a suppressor activity of IL-10 has been demonstrated in allergic subjects taking allergen sublingual immunotherapy and also in patients with respiratory allergy harboring *Schistosoma* infections. Peripheral blood mononuclear cells in these patients produce more IL-10 and less IL-5 when stimulated with allergens than do such cells from allergic subjects without this worm infection [21–25]. However, because most SLE patients used glucocorticoid, the down-regulation of allergic inflammation in

SLE may also involve this medication, which may increase the gene transcription of IL-10 indirectly and decrease the gene transcription of both IL-4 and IL-5. In addition, glucocorticoid may inhibit the expression of the IL-4 receptor and also of the high-affinity IgE receptor [26, 27].

We conclude that a vigorous autoimmune response of specific antinuclear IgE antibodies can be detected in some SLE patients, and that this response consists of au-

toantibodies of this isotype against self cellular components that have important biological roles in protein expression, cellular proliferation and cell death.

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