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*J Immunol* 2012; 189:1535-1539; Prepublished online 13 July 2012;  
doi: 10.4049/jimmunol.1200842  
<http://www.jimmunol.org/content/189/4/1535>

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## Cutting Edge: Brazilian Pemphigus Foliaceus Anti-Desmoglein 1 Autoantibodies Cross-React with Sand Fly Salivary LJM11 Antigen

Ye Qian,\* Joseph S. Jeong,\* Mike Maldonado,\* Jesus G. Valenzuela,<sup>†</sup> Regis Gomes,<sup>†</sup> Clarissa Teixeira,<sup>†</sup> Flor Evangelista,\* Bahjat Qaqish,<sup>‡</sup> Valeria Aoki,<sup>§</sup> Gunter Hans, Jr.,<sup>¶</sup> Evandro A. Rivitti,<sup>§</sup> Donald Eaton,<sup>¶</sup> and Luis A. Diaz\*

The environmental factors that contribute to the development of autoimmune diseases are largely unknown. Endemic pemphigus foliaceus in humans, known as Fogo Selvagem (FS) in Brazil, is mediated by pathogenic IgG4 autoantibodies against desmoglein 1 (Dsg1). Clusters of FS overlap with those of leishmaniasis, a disease transmitted by sand fly (*Lutzomyia longipalpis*) bites. In this study, we show that salivary Ags from the sand fly, and specifically the LJM11 salivary protein, are recognized by FS Abs. Anti-Dsg1 monoclonal autoantibodies derived from FS patients also cross-react with LJM11. Mice immunized with LJM11 generate anti-Dsg1 Abs. Thus, insect bites may deliver salivary Ags that initiate a cross-reactive IgG4 Ab response in genetically susceptible individuals and lead to subsequent FS. Our findings establish a clear relationship between an environmental, noninfectious Ag and the development of potentially pathogenic autoantibodies in an autoimmune disease. *The Journal of Immunology*, 2012, 189: 1535–1539.

It is an accepted assumption that the interaction of unknown environmental factors with susceptibility genes of the host causes the immune system to react to self-antigens, producing a spectrum of autoimmune diseases (1). The common thread among autoimmune diseases is the obscure etiology. Human organ-specific autoimmune diseases targeting the skin constitute the pemphigus group, in which pathogenic IgG4-restricted, anti-epidermal autoantibodies cause epidermal cell detachment that leads to blister formation (2). The Ag recognized by these autoantibodies in pemphigus foliaceus (PF) is desmoglein 1 (Dsg1). The idiopathic, nonendemic form of PF is known worldwide, whereas an endemic variety, Fogo Selvagem (FS), is seen in certain regions of subtropical Brazil (3). FS shows clinical, histological,

and immunological features similar to those of nonendemic PF, except for the unique epidemiology of FS. A case-control epidemiological study of FS in Brazil suggested that certain living conditions and exposure to hematophagous insect bites were risk factors of FS (4). Exposure to bites of three insects is thought to be linked to FS: *Lutzomyia longipalpis* (sand flies), reduviids (kissing bugs), and simuliids (black flies). They are vectors of leishmaniasis, Chagas disease, and onchocerciasis respectively. Moreover, the sera of a large number of FS patients possess anti-Dsg1 autoantibodies (5).

A recent study has demonstrated that not only IgM and IgG4 anti-Dsg1 autoantibodies are detected in the sera of FS, but also IgE (6). It is remarkable that IgG4 anti-Dsg1 autoantibodies are restricted and pathogenic in FS; however, the mechanisms involved in the emergence of these autoantibodies are completely unknown. The endemic nature of FS and the circumstantial evidence presented above allow us to test the hypothesis that salivary gland Ags from hematophagous insects are the source of sensitizing Ag that triggers the autoimmune disease in FS. We selected a well-defined system provided by *L. longipalpis*, in which the salivary gland proteins are well characterized (7, 8). In this investigation, we show that IgG4 and IgE autoantibodies from FS sera recognized salivary gland Ags from *L. longipalpis* (SGLL). The major SGLL antigenic component recognized by FS sera is LJM11. In addition, sera from mice immunized with LJM11 also recognize human rDsg1. These results strongly support the notion that LJM11 induces cross-reactive Abs in FS patients and experimental animals. This is the first evidence, to our knowledge, that a noninfectious agent may trigger a human autoimmune disease via molecular mimicry.

## Materials and Methods

### Serum samples and anti-Dsg1 mAbs from FS patients

FS sera ( $n = 45$ ) and two IgG4 anti-Dsg1 mAbs (4E4 and 2D11) derived from FS patients (9) were used. Sera from healthy controls (HC) ( $n = 43$ ) from the

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Received for publication March 20, 2012. Accepted for publication June 19, 2012.

This work was supported in part by National Institutes of Health Grants R01 AR30281, R01 AR32599 (to L.A.D.), and K01 AR056378 (to Y.Q.). Y.Q. was also supported

by a Dermatology Foundation research award and an American Skin Association Alice P. Melly research grant.

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Abbreviations used in this article: Dsg1, desmoglein 1; FS, Fogo Selvagem; HC, healthy control; IP, immunoprecipitation; PF, pemphigus foliaceus; RT, room temperature; SGLL, salivary gland Ag from *Lutzomyia longipalpis*.

University of North Carolina blood bank were included as controls (HC-University of North Carolina). Ten sera from normal donors living in Brazilian endemic areas of FS were included in some of the studies, as well (HC-endemic). This study was approved by the institutional review boards from the University of North Carolina, Chapel Hill, and the University of Sao Paulo, Sao Paulo, Brazil. The H and L chains of 4E4 and 2D11 (9) were cloned into the pComb3XSS vector and expressed in Top10 F' cells (10). A GST-tag was introduced to the 4E4 construct to increase the solubility of the recombinant 4E4 scFv, and 4E4-GST scFv was produced and purified by Genscript (Piscataway, NJ). The 4E4-GST scFv was not pathogenic when tested by passive transfer into neonatal mice (2) and the dispass assay (11), using concentrations up to 30 µg per dose and 5 µg/ml Ab, respectively.

#### Human rDsg1, sand fly salivary gland extract, and sand fly salivary proteins

The rDsg1 was generated and purified as described (12). SGLL and SGLL proteins LJM11, LJM17, and LJL143 were generated at the Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, by Valenzuela (8, 13).

#### ELISA

IgE and IgG4 anti-Dsg1 and anti-SGLL ELISAs were conducted as described in previous articles (6, 9, 14). The ELISA assay to detect IgG4 Ab activity against LJM11, LJM17, and LJL143 SGLL proteins was also conducted as above, with some modifications. Stripwell Microplates (Corning, Lowell, MA) were coated, 50 ng per well, with LJM11, LJM17, or LJL143 proteins. A 1:100 dilution of each of FS sera or scFv 4E4 and 2D11 anti-Dsg1 mAbs (50 ng/ml) was added and incubated. The bound IgG4 Abs from serum samples or scFv Abs were detected with anti-human IgG4 HRP (Zymed, San Francisco, CA) or anti-hemagglutinin–HRP conjugate (Roche, Indianapolis, IN), respectively. Anti-Dsg1 ELISA, using SGLL recombinant protein immunized mouse sera, was conducted according to the regular anti-Dsg1 ELISA described above, with the following modification. The mouse sera were diluted 1:500, and goat anti-mouse IgG (Fc fraction) HRP conjugate (Jackson ImmunoResearch, Carlsbad, CA) was used to detect anti-Dsg1 Abs from mouse sera.

#### Immunoprecipitation

Pan mouse IgG magnetic Dynabeads (Invitrogen, Carlsbad, CA) were used for immunoprecipitation (IP) according to the manufacturer's instructions, with modifications. Mouse anti-human IgG4 and mouse anti-GST-tag mAbs (Zymed, San Francisco, CA) were used for IP of sand fly salivary gland proteins LJM11, LJM17, and LJL143. All IPs were processed in TBS buffer containing 0.5% Tween 20 and 5 mM CaCl<sub>2</sub>, plus 0.1% BSA. First, the Dynabeads (20 µl slurry) were blocked with 0.1% BSA in TBS buffer containing 0.5% Tween 20 and 5 mM CaCl<sub>2</sub> for 1 h at room temperature (RT) and incubated with mouse anti-GST (for 4E4-GST) or mouse anti-human IgG4 (for serum samples) for 1 h at RT, and then 4E4-GST or human serum samples for 2 h at RT. Finally, Dynabeads were incubated with LJM11, LJM17, and LJL143 for 2 h at RT. For IP using SGLL recombinant protein-immunized mouse sera, 2 µl each mouse serum sample was incubated with 450 µl rDsg1 cell culture supernatant for 2 h at RT and then with 20 µl Dynabead slurry for 1 h. Each IP sample was subjected to SDS-PAGE and subsequent Western blot. The membranes were probed with anti-His HRP (Qiagen, Valencia, CA) and the bands revealed with chemiluminescent substrate (Pierce, Rockford, IL).

#### Statistical analysis

Groups were compared using the *t* test. Correlation analysis was accomplished with the Pearson correlation.

## Results and Discussion

### FS patients have IgE and IgG4 Abs against *L. longipalpis* salivary gland Ags

We have recently reported that FS patients possess significantly higher levels of IgE and IgG4 anti-Dsg1 Abs than do control groups from nonendemic areas of Brazil and the United States (6). It is possible that generation of these anti-Dsg1 autoantibodies may be secondary to exposure and sensitization to an environmental Ag or Ags. The sera from FS patients and healthy controls from the United States (HC-University of North Carolina) were tested for IgE and IgG4 Ab activity against SGLL by ELISA. As shown in Fig. 1A, the index values of IgE

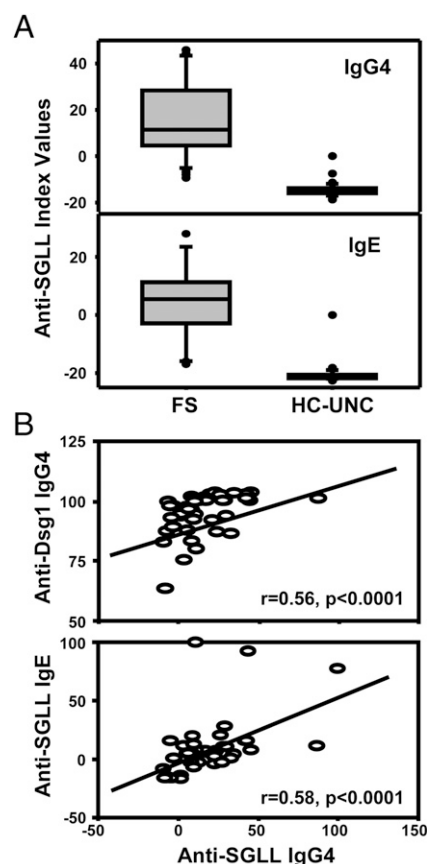


**FIGURE 1.** FS patients have high levels of anti-SGLL IgG4 and IgE Abs. (A) Boxplot analysis of the IgG4 and IgE responses in FS patients ( $n = 45$ ) and UNC healthy controls ( $n = 43$ ) against SGLL Ags by ELISA. The index values are higher in the FS group than in the HC-UNC group. (B) Pearson correlation analysis between the IgG4 anti-SGLL and IgG4 anti-Dsg1 responses (upper panel) and the IgE anti-SGLL and IgG4 anti-SGLL responses (lower panel) in the same donors of the FS group.

and IgG4 anti-SGLL Abs are significantly higher in FS patients ( $n = 45$ ) than in HC-University of North Carolina controls ( $n = 43$ ) ( $p < 0.001$ ). The IgG4 anti-SGLL Ab re-

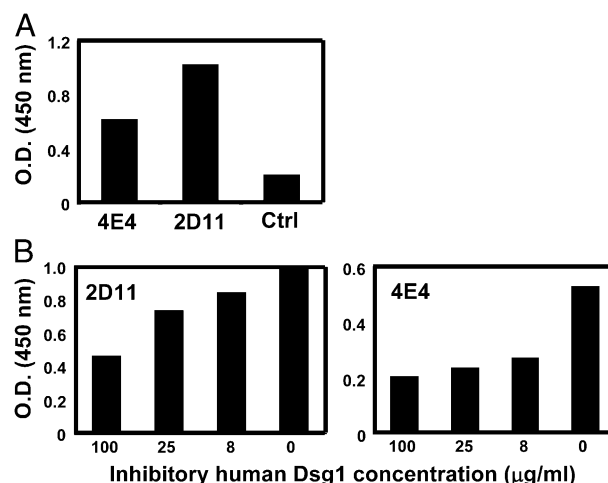


**FIGURE 2.** The cross-reactivity of two IgG4 anti-Dsg1 mAbs from FS to SGLL by ELISA. (A) Both IgG4 anti-Dsg1 mAbs (scFv 4E4 and scFv 2D11) react to SGLL. (B) The binding of both mAbs, 2D11 (left panel) and 4E4 (right panel), to SGLL is inhibited by Dsg1 in a concentration-dependent manner. These results were repeated three times with similar results.

sponse in the FS group was significantly correlated with both the IgG4 anti-Dsg1 (Pearson correlation,  $r = 0.56$ ,  $p < 0.0001$ ) and the IgE anti-SGLL ( $r = 0.58$ ,  $p < 0.0001$ ) (Fig. 1B). These findings strongly suggest that the generation of potentially pathogenic IgG4 anti-Dsg1 in FS may be associated with the antigenic stimulation produced by salivary Ags from hematophagous insects.

#### The cross-reactivity of anti-Dsg1 Abs to SGLL

To determine whether the anti-SGLL activity from FS sera is due to the cross-reactivity of anti-Dsg1 autoantibodies, ELISA was performed with two scFv IgG4 monoclonal anti-Dsg1 Abs against SGLL Ags, derived from two FS patients (9). As shown in Fig. 2A, both scFv recognize SGLL. To confirm the cross-reactivity of the two monoclonal anti-Dsg1 autoantibodies with anti-SGLL Abs, a competition ELISA assay was performed using human rDsg1 as an inhibitor of the binding of anti-SGLL Abs to immobilized SGLL Ag. As shown in Fig. 2B, binding of both mAbs to SGLL is inhibited in a dose-dependent manner by soluble Dsg1. These findings suggest that

the anti-SGLL Ab activity in FS patients is, at least in part, due to cross-reactivity of the anti-Dsg1 autoantibodies.

#### The LJM11 major immunogenic component of SGLL is recognized by FS sera and IgG4 monoclonal anti-Dsg1 Abs

A large number of secreted proteins are present in *L. longipalpis* saliva (7, 8, 13), and LJM11 and LJM17 are the two main Ags that incite Ab responses in humans. Other salivary Ags, such as LJL143, are weakly recognized by humans (13). These three recombinant proteins were chosen to determine whether they are recognized by IgG4 Abs from FS patients and normal controls from either FS endemic regions (HC-endemic) or a non-FS endemic region (HC-University of North Carolina). Ten serum samples from each group were randomly selected and tested. As shown in Fig. 3A, we found that the reactivity of FS sera was consistently much stronger to LJM11 than to LJM17 and LJL143. In addition, the level of anti-LJM11 Abs from FS patients is significantly higher than that from healthy donors from HC-endemic ( $t$  test,  $p = 0.00029$ ) and HC-University of North Carolina regions ( $t$  test,  $p = 0.00013$ ). These results show that IgG4 Abs from FS sera chiefly recognize the LJM11 component of SGLL.

We next tested the reactivity of 4E4 and 2D11 IgG4 anti-Dsg1 mAbs against LJM11, LJM17, and LJL143. As shown in Fig. 3B, both mAbs bind strongly to LJM11, but weakly to LJM17 and LJL143. These findings are similar to those observed when testing FS sera. To further confirm that these two mAbs cross-react to LJM11, an ELISA inhibition assay was conducted. A dose of 100  $\mu$ g/ml of rDsg1 incubated with these Abs was able to inhibit 90% and 70% of the binding of 4E4 and 2D11 to LJM11, respectively (Fig. 3C). In addition, IP using mAb 4E4 and FS patient serum shows that both

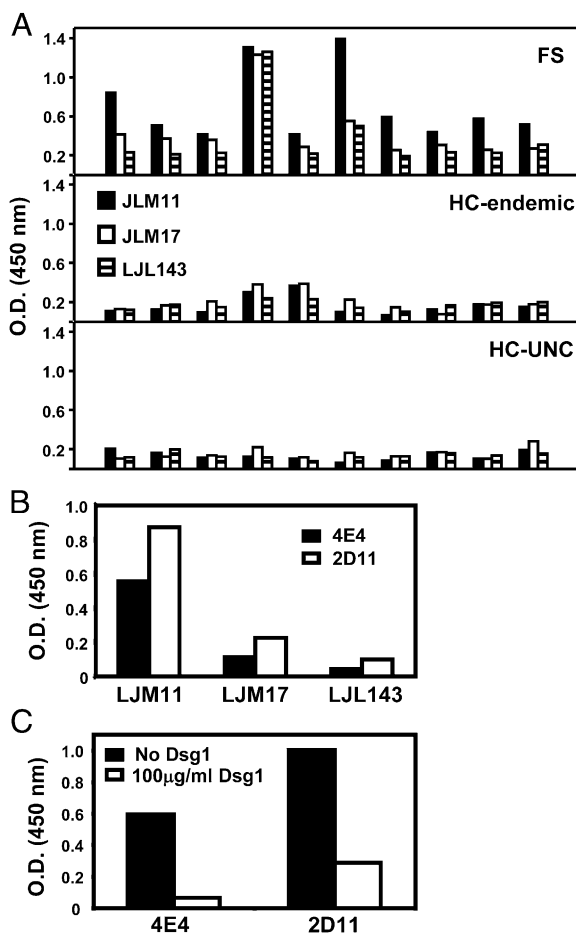


**FIGURE 3.** IgG4 Abs from FS patients and two IgG4 monoclonal anti-Dsg1 Abs derived from FS patients recognize LJM11, a protein component from SGLL. (A) The reactivity of ten randomly selected FS sera (top panel) is higher than that in HC-endemic donors (middle panel) and HC-UNC donors (bottom panel) when tested by ELISA with three SGLL main proteins, LJM11, LJM17, and LJL143. The reactivity is mainly with the LJM11 protein. The difference between HC-endemic and HC-UNC groups by the Student  $t$  test is not significant ( $p = 0.3800$ ). (B) The 4E4 and 2D11 IgG4 monoclonal anti-Dsg1 Abs also recognize the LJM11 component from SGLL. (C) The binding of both anti-Dsg1 mAbs to LJM11 is inhibited by Dsg1 protein.

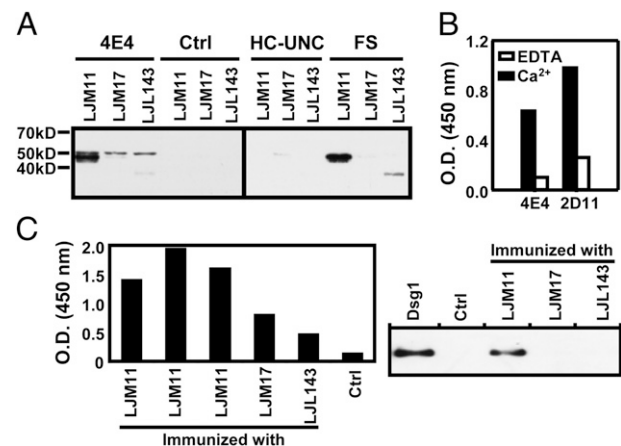


**FIGURE 4.** FS autoantibodies recognize LJM11, and sera from mice immunized with LJM11 bind human Dsg1. (A) The 4E4 anti-Dsg1 mAb (lanes 1–3) and control samples without 4E4 (lanes 4–6) (left panel), and HC-UNC (lanes 7–9) and FS serum (lanes 10–12) (right panel) were tested by IP against LJM11, LJM17, and LJL143 Ags. The LJM11 heavy band is precipitated by 4E4 anti-Dsg1 mAb and FS serum. (B) The reactions of 4E4 and 2D11 anti-Dsg1 mAbs with LJM11 by ELISA in the presence of 5 mM Ca<sup>2+</sup> (black columns) are significantly inhibited in the presence of 5 mM EDTA (white columns). (C) Three sera from mice immunized with LJM11 show strong reactivity to human Dsg1 by ELISA, compared with sera from mice immunized with LJM17 and LJL143 and control mice (left panel). The right panel shows human Dsg1 (lane 1), control murine serum (lane 2), mouse anti-LJM11 (lane 3), mouse anti-LJM17 (lane 4), and mouse anti-LJL143 (lane 5) tested against human Dsg1 by IP. The only serum that immunoprecipitates human Dsg1 is that from mouse anti-LJM11.

react strongly to LJM11 (~43 kDa), but weakly to LJM17 (~45 kDa) and LJL143 (~34 kDa) (Fig. 4A). These findings suggest that LJM11 is the main component of SGLL that triggers the IgG4 immune response in humans and that cross-reacts with Dsg1 autoantigen.

*The binding of anti-Dsg1 mAbs to LJM11 is conformational and Ca<sup>2+</sup> dependent*

Both 4E4 and 2D11 anti-Dsg1 mAbs failed to react with denatured LJM11, LJM17, and LJL143 by Western blot analysis (data not shown), suggesting that 4E4 Ab binding to the SGLL is conformational. Because binding of pathogenic autoantibodies to Dsg1 is conformational and Ca<sup>2+</sup> dependent (15), we tested the reactivity of these two Abs to LJM11 in the presence of either Ca<sup>2+</sup> or EDTA. As shown in Fig. 4B, the binding of these mAbs from FS patients to LJM11 is also Ca<sup>2+</sup> dependent. These results suggest that the epitopes recognized by FS sera on SGLL, like those on Dsg1, are Ca<sup>2+</sup> dependent and conformational.

*Mice immunized with LJM11 produce anti-Dsg1 Abs*

If SGLL proteins introduced by sand fly bites induce Abs that cross-react to both human Dsg1 and LJM11 in humans, it is expected that mice immunized with LJM11 also generate cross-reactive Abs to human Dsg1. Anti-Dsg1 reactivity of the sera from these mice and control mice was tested by ELISA. As shown in Fig. 4C (*left panel*), sera from all three mice immunized with LJM11 strongly react to human Dsg1. The Dsg1 reactivity of these mouse sera was also tested by IP. As shown in Fig. 4C (*right panel*), the serum from a mouse immunized with LJM11 Ag also immunoprecipitates human Dsg1. Control serum and sera from mice immunized with LJM17 and LJL143 Ags do not show reactivity. These findings further confirm that LJM11 is the major component in SGLL that induces the cross-reactive Abs in both human FS patients and experimental mice.

It is unusual that a population of Abs would develop cross-reactivity to two evolutionarily distant molecules, the human Dsg1 and a sand fly salivary gland component. No amino acid sequence similarity exists between Dsg1 and LJM11 (data not shown). The Ca<sup>2+</sup>-dependent interaction suggests that the autoantibodies react with both molecules via conformational, but not linear, epitopes. This idea could explain how two evolutionarily distant molecules can both be recognized by cross-reactive Abs because conformational epitopes of both molecules may be the same, even though their linear structures do not show significant homology.

Our findings are also in line with molecular mimicry as the mechanism for how environmental factors induce autoimmune diseases (1, 16). Unlike infectious agents that induce robust IgG immune responses, the low dose of the Ags and the route of the exposure (skin) introduced by insect bites predictably induce an IgE response (17). It is known that endemic regions of FS in Brazil are heavily infested with sand flies, and individuals living in these areas are constantly exposed to these pests. It is likely that these individuals mount IgE and IgG4 responses to salivary Ags, such as the LJM11 protein. Similar Ab responses have been described in people frequently stung by bees or during immunotherapy of allergic patients (18–21). It would be expected that those genetically predisposed individuals (22) living in endemic areas of FS are

constantly exposed to sand fly bites (and would be sensitized to salivary Ags, including the LJM11 protein), thus generating IgG4 and IgE anti-SGLL Abs, which, as shown in this article, may cross-react with unique epitopes on the ectodomain of human Dsg1. Epitope spreading, as previously reported (23), may be the underlying mechanism that leads to the generation of a more diverse autoantibody response in FS. The cross-reactive Abs may constitute a complex population of non-pathogenic and perhaps pathogenic Abs. In this context, it would be feasible that certain FS IgG4 autoantibodies may exhibit distinct epitope specificity with those IgG4 anti-SGLL Abs that cross-react with human Dsg1. It is clear, however, that testing the pathogenicity of these cross-reactive Abs is in need of further investigation. In summary, this investigation provides the first direct evidence, to our knowledge, that a non-infectious environmental agent may play a significant role in initiating an autoimmune disease via molecular mimicry.

## Disclosures

The authors have no financial conflicts of interest.

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