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# Overlapping IgG4 Responses to Self- and Environmental Antigens in Endemic Pemphigus Foliaceus

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The etiology of human autoimmune diseases in general remains largely unknown, although the genetic and environmental interplay may be relevant. This applies to the autoimmune diseases of the skin such as the pemphigus phenotypes and others. In this group, there is an endemic form of pemphigus foliaceus (also known as fogo selvagem [FS]) in which the pathogenic IgG4 autoantibody response to the self-antigen desmoglein 1 (Dsg1) cross-reacts with the LJM11 sand fly salivary gland Ag. In this investigation, we dissected the IgG4 autoantibody repertoires used by FS patients in response to endogenous self-Dsg1 and exogenous LJM11 sand fly Ag. Based on analyses of the genetic clonal signatures of these Abs, our results indicate that there is a significant overlap between these two responses, as all identified IgG4 mAbs cross-react to both Dsg1 and LJM11 Ags. Germline H- and L-chain V gene Abs generated according to mutated cross-reactive mAbs preserved their reactivity to both Ags. Our findings suggest that both Dsg1 autoantigen and LJM11 environmental Ag could be the initial antigenic stimulants for the IgG4 autoimmune responses in FS. These results support our hypothesis that LJM11 Ag plays a substantial role in triggering the IgG4 autoantibody development in FS and provide new insights on how noninfectious environmental Ag(s) may drive the generation of autoantibodies in IgG4-related autoimmune diseases. *The Journal of Immunology*, 2016, 196: 2041–2050.

**F**ogo selvagem (FS) is an endemic form of pemphigus foliaceus (PF) found in certain states of Brazil (1, 2). The hallmark of this disease is the presence of intraepidermal vesicles due to epidermal cell detachment (acantholysis) (3) induced by pathogenic IgG4 anti-desmoglein 1 (Dsg1) autoantibodies (4–8). FS shows similar clinical, histological, and immunological features to those observed in nonendemic PF (9, 10). Epidemiological and immunogenetic studies suggest that both genetic and environmental factors contribute to the development of FS (1, 11, 12). Previous studies suggest that exposure to hematophagous insect bites in genetically predisposed individuals may be a risk factor for FS (12). To strengthen this hypothesis, we have shown that IgG4 anti-Dsg1 autoantibodies cross-react with LJM11 sand fly salivary gland Ag (13), which suggests that the development of IgG4 Abs may be linked to immune responses to environmental Ags. Com-

pared to investigations on the pathogenesis and genetic predisposition of autoimmune diseases, etiological studies regarding environmental triggers of these diseases are lacking due to low prevalence and the clinical heterogeneity of the diseases (14–19). Similarly, the random nature of autoimmune skin diseases in North America makes it difficult to assess their etiological commonality and further dissect their causes. In this regard, the endemic nature of FS provides an invaluable model and rare opportunity to study the environmental factors within the FS endemic region and their contribution to the development of FS.

IgG4 Abs are known to be elevated in patients with FS (20–22) and other bullous dermatoses (23), as well as autoimmune pancreatitis (24), Mikulicz's disease (primary Sjögren's syndrome) (25), and other diseases (26). Recently, the terms IgG4-related disease and IgG4-related skin disease have been proposed (26–28). Among some autoimmune diseases, increased serum levels of total IgG4 are often observed, and certain specific histopathological features, such as IgG4 plasma cell infiltration in effected tissues or organs, are present (26–28). In contrast, increased circulating anti-Dsg1 IgG4 autoantibodies are characteristic of FS/PF, as these anti-epidermal autoantibodies are pathogenic and detected bound to the surface of detached keratinocytes in lesional and perilesional epidermis of these patients (4). In FS/PF, the lesional skin does not show IgG4 B cell or plasma cell infiltrates. In 1989, Rock et al. (20) demonstrated that the IgG4 response in FS is pathogenic. Later studies confirmed that the bulk of pathogenic anti-Dsg1 autoantibodies in FS are predominantly IgG4 (21). IgG4 anti-Dsg1 Abs from FS patients are pathogenic in mice (8, 29), similar to those in PF (30) using an IgG passive transfer mouse model. One study showed that progression from the pre-clinical to the clinical stage of the disease is associated with a dramatic rise in IgG4 anti-Dsg1 autoantibodies (21) and that the level of anti-Dsg1 IgG4 Abs can be used as a predictor of FS (22). Our recent finding that IgG4 autoantibodies in FS cross-react with an LJM11 sand fly Ag (13) suggests that the development of IgG4 autoantibodies in patients may be linked to exposure to an envi-

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Abbreviations used in this article: APD, Ab phage display; Dsg1, desmoglein 1; FS, fogo selvagem; FWR, framework region; PF, pemphigus foliaceus; PV, pemphigus vulgaris; R/S, replacement and silent mutations; scFv, single-chain fragment variable.

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ronmental Ag. The studies of the association of environmental factors, such as infectious agents, with the development of autoimmune diseases have a long history (15, 17–19, 31–34). However, the studies of noninfectious Ags and their association with the development of autoimmune diseases are scarce. Because sand flies are prevalent in FS endemic areas, it is possible that individuals living in these endemic regions, especially among those FS-susceptible individuals, may generate an immune response to Ags from sand fly saliva (i.e., LJM11). The LJM11 Ag, a member of the yellow family of salivary proteins, is one of the most immunogenic component among the sand fly saliva proteins and also a marker for human exposure of sand fly bites (35). That anti-LJM11 Abs cross-react to autoantigen Dsg1 (13, 36) strongly suggests the existence of a cross-reactive epitope(s) on both LJM11 and Dsg1. Because exposure to exogenous Ags may induce the autoimmune response to cross-reactive autoantigens in autoimmune diseases (33, 34, 37–39), we hypothesize that the immune response directed against this cross-reactive epitope(s) subsequently initiates the generation of IgG4 autoantibodies in FS-susceptible individuals.

The aim of this investigation was to determine if the IgG4 autoantibody responses to Dsg1 and LJM11 sand fly Ags overlap at the Ab V gene level on FS patients from endemic areas. Our study indicates that there is a striking overlap between these two IgG4 responses and LJM11 could be an inciting Ag for autoantibody development, strongly suggesting that LJM11 sand fly Ag plays an important role in the etiology of FS.

## Materials and Methods

### *Immortalized B cell samples from FS patients*

Immortalized B cell samples from FS patients in this investigation have been described before (40) and kept at the University of North Carolina Dermatology Research Laboratories. These studies are approved by the institutional review boards from the University of North Carolina at Chapel Hill.

### *IgG4 Ab phage display library construction*

To amplify the VH and VL genes for Ab phage display (APD) library construction, mRNA from the immortalized B cells of three FS patients (13) were isolated, and their cDNA were synthesized. The L-chain V genes were amplified as originally described (41). To obtain IgG4 only H-chain genes, we used the 5' primers described from the original method (41), whereas the 3' primers for VH genes from the original method (41) were replaced by an IgG4-specific primer based on an IgG4 Ab C-region-specific sequence designed by Efremov et al. (42). A 209-bp DNA segment from the H-chain C-region (including 36 bp from the IgG4 hinge region) was amplified along with the H-chain V genes transcripts. The resulting IgG4 single-chain fragment variable (scFv) comprises L- and H-chain V segments and partial C-region of IgG4 H-chain (Supplemental Fig. 1). The subsequent APD library construction was according to the original method described (41). To introduce FLAG-tag at the N-terminal of the resulting scFv for more convenient detection and purification, the original pComb3xSS vector was modified. The primer sequences used for PCR are: 5'–ATAGGCCAGGCCGCGCCAGCACCATCACCATCACCATTAGGAG–GGTGGTGGCTCTG–3' (for removing HA at C-terminal) and 5'–ATAGGCCGCTGGGCCAACTTGTCATCGTCATCCTTGTAATCCGCA–GCTTGGGCCACGGTAGCGAA–3' (for adding FLAG at N-terminal). PCR cycle consisted of: 94°C, 4 min for one cycle; 94°C for 30 s/55°C for 30 s/72°C for 5 min for 30 cycles; and 72°C for 10 min for one cycle. This PCR product was SfiI digested and cloned to modified pComb3xSS vector (with FLAG-tag at the N-terminal).

### *Isolation of anti-Dsg1, anti-LJM11, and cross-reactive mAbs*

The constructed IgG4 APD libraries were panned as described (41) against either Dsg1 or LJM11 for three rounds or the alternation of Dsg1 and LJM11 for total of four rounds (Supplemental Table I). These selected clones were amplified and their H- and L-chain sequenced. Selected clones were subjected to scFv protein expression and production. Produced scFv mAbs were purified by either Nickel column (Qiagen) or anti-FLAG magnetic beads (Sigma-Aldrich, St. Louis, MO) according to the manufacturers' instruction for further testing. Human Dsg1 and sand fly LJM11 protein were purified as described (13, 35, 36, 40, 43).

### *ELISA and other mAbs*

The specificity of those purified scFv mAbs were tested by ELISA as previous described (13, 36, 40, 44). Negative control scFv (TT1) was kindly provided by Drs. Don Siegel and Stephen Kacir from the University of Pennsylvania and subcloned in our modified pComb3xSS vector with an N-terminal FLAG-tag. Anti-FLAG-HRP (Sigma-Aldrich) was used as secondary Ab for detection of all purified scFv mAbs.

### *Revertant scFv mAbs*

The germline sequences of the H- and L-chain of two mAbs (4E4 and 2D11) isolated previously (40) were identified using The National Center for Biotechnology Information (IgBlast) (45). The H- and L-chain V genes of the germline formats of these two mAbs were synthesized (GenScript, Piscataway, NJ) and subcloned into our modified pComb3xSS vector. The revertant scFv mAbs (4E4-R and 2D11-R) and their mutated counterpart scFv mAbs (4E4 and 2D11) were expressed and purified as described (41).

### *VH gene analysis*

The identified scFv mAb H-chain genes were analyzed using Ab gene database at The National Center for Biotechnology Information (IgBlast) (45). Vector NTI (Invitrogen) is used for multiple alignments of the VH genes.

### *Statistics*

The significant difference between the ratio of replacement and silent mutations (R/S) for CDRs and framework regions (FWRs) is assessed using  $\chi^2$  test.

## Results

### *Construction of IgG4 APD libraries from FS B cells*

Because the main pathogenic autoantibodies are of the IgG4 subclass in FS (20, 21), we sought to focus on the characterization of IgG4 Abs from FS patients. The APD library constructed using the method originally described by Barbas et al. (41) includes the Ab repertoire from all four IgG subclasses in a constructed IgG phage display library, and the original IgG subclass identity could not be determined from resulting scFv Abs from the library. However, because of the predominant presence of IgG4 Abs in autoimmune skin diseases (4–8) and other IgG4-related diseases (26), the identification and isolation of IgG4 mAbs from these patients became increasingly necessary to understand the mechanism of IgG4 Ab development in these patients. Thus, we have modified the method for our particular purpose to obtain only IgG4 mAbs (Supplemental Fig. 1). The advantages of the resulting mAbs are that: 1) the scFv Abs in these libraries derived from FS B cells comprise IgG4 Abs only; and 2) the scFv contain partial constant and hinge regions of IgG4 immediately following VH domain, and their IgG4 subclass identity can be further confirmed. Employing this strategy, we have constructed three IgG4 phage display libraries using mRNA isolated from three FS patients' EBV-immortalized B cells.

### *Isolation of anti-Dsg1 and anti-LJM11 IgG4 mAbs by panning against Dsg1 and LJM11*

We have shown that FS patients have significantly higher IgG4 anti-Dsg1 and LJM11 Abs compared with normal controls from both FS (Lima Verde in Brazil) and non-FS endemic region (36), suggesting that there are IgG4 immune responses against both the autoantigen and environmental Ag. Additionally, that two anti-Dsg1 IgG4 mAbs isolated from FS patients previously using hybridoma method cross-react to both Dsg1 and LJM11 (13) suggests the presence of cross-reactive epitope(s). Thus, it is possible that IgG4 Abs are present in FS patients reacting to both Dsg1 and LJM11.

To determine whether there is an overlap between these anti-Dsg1 and anti-LJM11 IgG4 responses, we identified the anti-Dsg1 and anti-LJM11 IgG4 Abs from the IgG4 APD libraries

constructed from B cells of 3 FS patients to study the IgG4 Ab repertoires by panning against either Dsg1 and/or LJM11 (Supplemental Table I) (41). Colonies were randomly selected from Dsg1 and LJM11 panning of these three IgG4 APD libraries. Their plasmids were purified and subject to sequencing using Ompseq and HRML-F primers as described (41). The Ab H- and L-chain gene sequencing results of selected Ab clones from IgG4 APD libraries were analyzed using Ab gene database at The National Center for Biotechnology Information (IgBlast) (45) and their four FWRs and three CDRs determined. Totals of 39 (GCDS), 58 (FGS), and 39 (JLDO) clones from Dsg1 and LJM11 panning were successfully sequenced, and their VH and VL gene usages were determined. The additional H-chain C-region sequences of these mAbs were also analyzed and confirmed to be of IgG4 subclass, indicating that only IgG4 mAbs were identified from our constructed IgG4 APD libraries.

*Same parental B cell origins by IgG4 mAb clones isolated from panning of IgG4 APD libraries against either Dsg1 autoantigen or LJM11 environmental Ag*

The H-chain V regions of mAbs from pemphigus are known to contribute most of the Dsg binding. For example, Di Zenzo et al. (46) demonstrated that H-chain V gene somatic mutations are required for mAbs to maintain its binding to Dsg3, whereas L-chain V gene mutations are not required for their binding to Dsg3. Studies from the Stanley group indicated that the H-chain of an anti-Dsg mAb could pair with L-chains encoded by different VL genes without losing its Dsg specificity (47–50). Consistent with all of these previous findings, we also found that most of independent mAbs identified from either Dsg1 or LJM11 panning of FS IgG4 APD libraries have different L-chains paired with the same H-chains (data not shown). Therefore, all of our genetic analyses of these

mAbs in this investigation are centered on the H-chain V gene segments. Among all three CDRs, the CDR3 region is the most diverse part of the Ab V regions, and a unique CDR3 sequence represents the genetic clonal signature for a group of Abs derived from the same parental B cell. Based on their H-chain V gene usage and the clonal signature of clonally related Abs (51), these clones can be grouped to four, six, and four independent clones from three FS patients, respectively (Table I). It should be noted that the VH genes of two IgG4 mAbs (GCDS-2D11 and FGS-4E4) that were isolated using hybridoma method from our previous study (40) are also identified from these clones isolated in our current study. The H-chain of GCDS-2D11 is encoded by IGHV2-5, whereas that of FGS-4E4 is encoded by IGHV3-48. Both clusters of mAbs encoded by these two VH genes and their respective CDR3s, are identified from their corresponding IgG4 APD libraries (both VH genes are in boldface in Table I). It indicates that Ag-specific mAbs can be identified using both hybridoma and APD libraries, further validating our newly developed method for construction and panning of IgG4 APD libraries to identify only IgG4 mAbs.

We next used two methods to further determine whether these mAbs are cross-reactive to Dsg1 and LJM11. These three IgG4 APD libraries were panned against the alternation of Dsg1 and LJM11 for total four rounds (panned against Dsg1 for first and third rounds and panned against LJM11 for second and fourth rounds) (Supplemental Table I) to identify cross-reactive mAbs. Those identified clones were sequenced and compared with those clones identified from Dsg1 or LJM11 individual panning. Most mAbs found from Dsg1- and LJM11-only panning are also found from the Dsg1/LJM11 alternate panning and thus determined to be cross-reactive. Some representative scFv were produced and purified as described (41) and tested by both anti-Dsg1 and LJM11

Table I. IgG4 scFv isolated from panning of IgG4 APD libraries

| Patient | VH                             | CDR3 (FWR4)                                                  | Clones Identified from Panning |       | Cross-Reactivity Confirmed by         |
|---------|--------------------------------|--------------------------------------------------------------|--------------------------------|-------|---------------------------------------|
|         |                                |                                                              | Dsg1                           | LJM11 |                                       |
| GCDS    | <b>IGHV2-5*02<sup>a</sup></b>  | AHRRLLGLRYCSTSSCFG <b>DFDY<sup>b</sup></b><br>(WGQGTTLTVSS)  | 3                              | 1     | ELISA <sup>d</sup>                    |
|         | IGHV3-9*01                     | VKSDSTGSSIRDASIFDS<br>(WGQGSLLTVSS)                          | 9                              | 9     | Alternate panning, <sup>e</sup> ELISA |
|         | IGHV3-53*04                    | ARSLLSVGRYFDL(WGRGTLTVSS)                                    | 0 <sup>c</sup>                 | 2     | ELISA                                 |
|         | IGHV5-51*03                    | ARSYNSSE <b>YYIWFEA</b> (WGQGTTLTVSS) <sup>b</sup>           | 11                             | 4     | Alternate panning, ELISA              |
| Total   |                                |                                                              | 23                             | 16    |                                       |
| FGS     | IGHV1-18*01                    | ARDRNMRGLINKK <b>FYYYYGMDV<sup>b</sup></b><br>(WGQGTTLTVSS)  | 4                              | 0     | ELISA                                 |
|         | IGHV1-69*09                    | ASNNLTGTTVTYSYGMVDV<br>(WGQGTTLTVSS)                         | 2                              | 0     | ELISA                                 |
|         | <b>IGHV3-48*04<sup>a</sup></b> | ARGRTTFGGEGQLFDY<br>(WGQGTTLTVSS)                            | 9                              | 9     | Alternate panning, ELISA              |
|         | IGHV4-34*01                    | ARGLDFRSGYSSSDGDYDSYYVDV<br>(WGKGTTTVSS)                     | 7                              | 8     | Alternate panning, ELISA              |
|         | IGHV4-59*08                    | AGGYCNSTSCYNQAFYS <b>YYYYVDL<sup>b</sup></b><br>(WGTGTTTVSS) | 2                              | 4     | ELISA                                 |
|         | IGHV7-4-1*02                   | AKGGDYGDFD(WGPGTLTVSS)                                       | 7                              | 6     | Alternate panning, ELISA              |
|         | Total                          |                                                              | 31                             | 27    |                                       |
|         | JLDO                           |                                                              |                                |       |                                       |
| JLDO    | IGHV1-18*01                    | ARDLPTVVTPAPFDY(WGQGTTLTVSS)                                 | 3                              | 3     | Alternate panning, ELISA              |
|         | IGHV1-69*09                    | ASPYSGYDFLWAY(WGQGTTLTVSS)                                   | 5                              | 2     | ELISA                                 |
|         | IGHV3-23*01                    | AKWDHYPTAFDI(WGQGTMTVPS)                                     | 12                             | 6     | Alternate panning, ELISA              |
|         | IGHV4-34*01                    | ASRPLYCSGGNCYSYYQYGMNV<br>(WGQGTTLTVSS)                      | 5                              | 3     | Alternate panning, ELISA              |
| Total   |                                |                                                              | 25                             | 14    |                                       |

<sup>a</sup>Those mAbs in the VH column presented in bold were also identified previously using hybridoma method (40).

<sup>b</sup>The D/ExxxW amino acid motif and YYY aromatic amino acid clusters in CDR3 of four independent clones are in boldface.

<sup>c</sup>Those mAbs in italics were only identified from either Dsg1 or LJM11 panning.

<sup>d</sup>The cross-reactivity of these mAbs was confirmed by anti-Dsg1 and anti-LJM11 ELISA.

<sup>e</sup>The cross-reactivity of these mAbs was confirmed by Dsg1/LJM11 alternate panning.

ELISA (40, 44). All ELISA tested mAbs are cross-reactive (Table I and data not shown). Three independent clones isolated from either Dsg1 or LJM11 individual panning (one from GCDS and two from FGS; in italics in Table I) also cross-react to both Dsg1 and LJM11. Thus, all of the clones identified from our panning were cross-reactive based on one or the combination of two methods, and the results are summarized in Table I. Accordingly, despite differences in their mutations in CDRs and FWRs of those mAbs in each individual clonal lineage (see below and Fig. 1), their specificity to both Dsg1 and LJM11 is conserved.

#### Clonal selection and expansion among anti-Dsg1/LJM11 mAbs

Studies on some autoimmune diseases suggest that the autoimmune response in these diseases involves clonal selection and expansion events and is often oligoclonal (52, 53). In the present investigation, we isolated a total of 14 independent clones from 136 Dsg/LJM11-specific clones. It appears that autoimmune response within each individual patient is oligoclonal because only a limited number of independent clones were isolated. Some of the mAbs used the same VH genes by different FS patients. For example, IGHV1-18, IGHV1-69, and IGHV4-34 are shared by patients FGS and JLDO, with different CDR3 sequences between each lineage of mAbs from these two patients (Table I). Some of the mAbs from two patients share the same VH genes, suggesting there is a level of constrained VH gene usage in FS, consistent with other studies on the Ab repertoire in autoimmune diseases (53). The analyses of VH genes of these mAbs identified from panning of IgG4 APD libraries also demonstrate that the clonal expansion is apparent in all three patients. Phylogenetic trees in Fig. 1 suggest that evolutionarily these clones originated from the same parental B cells. For example, in the *top left panel*, all of

these Abs from patient GCDS are all encoded by IGHV5-51 VH gene and have the same CDR3 sequence. The VH genes of these Abs originated from the germline gene IGHV5-51 and underwent somatic mutation and clonal expansion during their maturations (see below). It is apparent that there is no divergent development among these mAbs identified from panning against either Dsg1 (clones initiated with D in Fig. 1) or LJM11 (clones initiated with L in Fig. 1). Most of the Dsg1 and LJM11 clones can be found on the same branches, further suggesting that Dsg1 and LJM11 have shared epitope(s), and both may serve as the selecting Ag for driving the development of these Abs.

It is interesting that the H-CDR3 of two independent clones from one patient (GCDS) have D/ExxxW consensus sequences (in boldface in Table I), which is the same as shared amino acid sequences in pathogenic pemphigus mAbs (50). Two H-CDR3 of independent clones from another patient also have YYY aromatic amino acid clusters (in boldface in Table I), similar to a pathogenic mAb from another study (46).

#### Extensive mutations among anti-Dsg1/LJM11 IgG4 Abs in FS patients

As it has been shown, those mutations are not the results of PCR errors through amplification of H- and L-chain V genes during APD library constructions (47). To further determine whether there are any characteristic mutations of anti-Dsg1 and anti-LJM11 IgG4 Abs in FS during their evolution, the somatic mutations of five lineages of mAb clones were identified using IgBlast and aligned (Fig. 2). All mAbs isolated from the libraries are extensively mutated, both in their CDRs and FWRs (Fig. 2). Similar to those phylogenetic analyses shown in Fig. 1, the different mutations among different clones in the same lineages of mAbs (using the same VH genes) demonstrate clear stepwise mutation during their

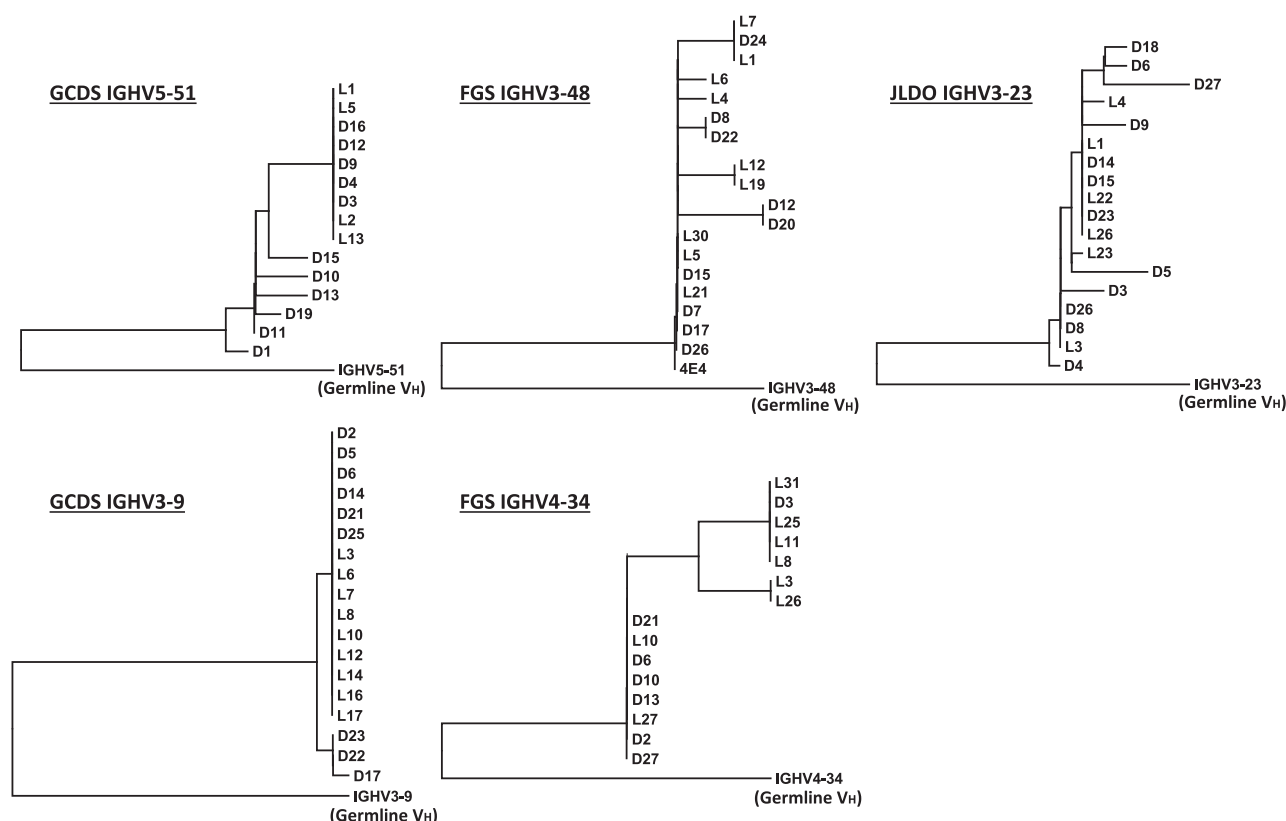


**FIGURE 1.** Phylogenetic tree of clonally related IgG4 Abs isolated from panning of IgG4 ADP libraries. Clone names starting with D indicate these clones were identified from Dsg1 panning, whereas those starting with L indicate they were identified from LJM11 panning.

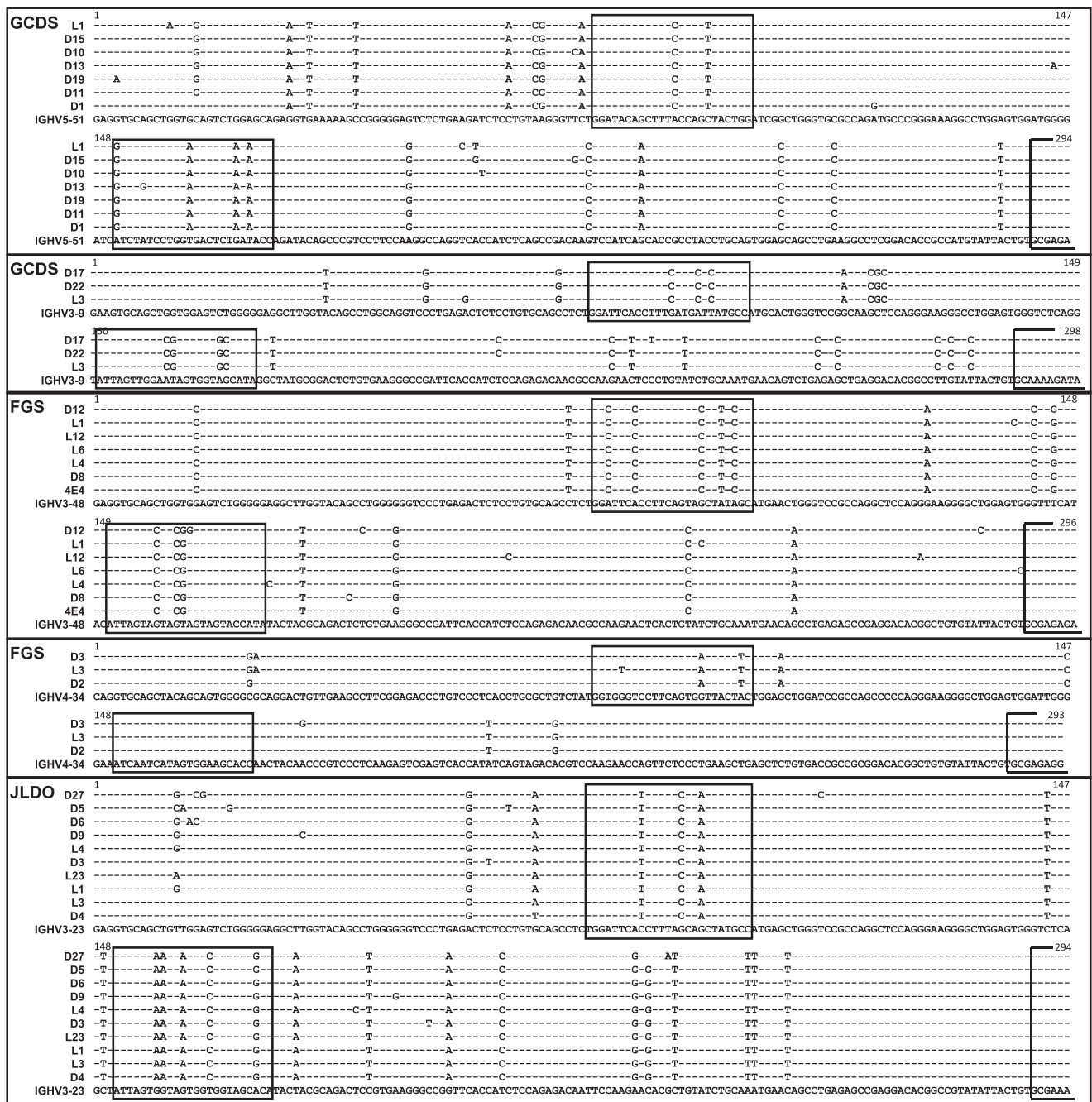


**FIGURE 2.** Alignments of clonally related IgG4 Abs identified from IgG4 ADP libraries. CDR1, CDR2, and partial CDR3 sequences are boxed. Mutated nucleotides are noted above the germline sequences. One representative clone is selected for those clones that have the identical VH gene sequences.

evolutions, consistent with continuous antigenic stimulation. One striking consistency is that despite extensive different FWR mutations among those different clones of the same clonal lineage, almost all of the mutations at CDR regions are preserved. For example, clones from IGHV3-23 lineage of JLDO shown in Fig. 2 (bottom panel), all mutations at CDR1 and CDR2 (boxed) are identical (C63T, G89C, and G92A in CDR1 and G15157A, G158A, G161A, T165C, and A172G in CDR2). In contrast, there is at least one mutation difference at FWRs for these listed clones using the same VH gene (IGHV3-23). In addition, as shown in Fig. 3 using GCDS-L3 as an example, except one mutation at CDR2 (T-C mutation), all other mutations at CDR1 and CDR2 are replacement mutations, which result in the change of amino acids compared with the germline VH (IGHV3-9). Those highly preserved replacement mutations at

CDRs suggest that they were specifically favored during affinity maturation of these mAbs during their evolution. The average ratios of R/S in CDRs are 2.47, 2.27, and 7.92, respectively, for the three FS patients, which is significantly higher than that in FWR (1.08, 1.17, and 1.26) ( $\chi^2$  test,  $p < 0.001$ ) (Table II). The CDR R/S ratios of 10 clones (in boldface in Table II), out of a total of 14 independent clones, are  $>2.9$ , which also indicates Ag selection (54). Highly diverse nucleotide mutations on the same VH clone clusters suggests that these mAbs underwent multiple rounds of clonal expansion, consistent with our hypothesis that chronic antigenic stimulation (by either self-antigen or environmental Ag, or both) is a feature for FS Ab development.

Shared mutations have been found previously in CDRs of the same VH genes encoding anti-Dsg1 mAbs among FS patients and

|                |                                                                                             |                        |               |
|----------------|---------------------------------------------------------------------------------------------|------------------------|---------------|
| <b>GCDS-L3</b> | E S G G G L V Q P G R S L R L S C A G S                                                     | <b>G F T F H A H A</b> | M H           |
|                | GAGTCTGGGGGAGGCTTGGTTTCAGCCTGGCAGGTGCGTGAGGCTCTCCTGTGCAGGCTCTGGATTACCTTTTCATGCTCATGCCATGCAC |                        |               |
| <b>IGHV3-9</b> | .....A.....C.....A.....C.....G...A.T.....                                                   |                        |               |
|                | E S G G G L V Q P G R S L R L S C A <u>A</u> S                                              | <b>G F T F D D Y A</b> | M H           |
| <b>GCDS-L3</b> | W V R Q R P G K G L E W V S G                                                               | <b>I S W T S G A I</b> | G Y A D S V K |
|                | TGGGTCCGACACGCCAGGGAAGGGCCTGGAGTGGGTCTCAGGTATATAGTTGGACGAGTGGTGCCATAGTTATGCGGACTCTGTGAAG    |                        |               |
| <b>IGHV3-9</b> | .....G...GCT.....AT.....AG.....C.....                                                       |                        |               |
|                | W V R Q <u>A</u> P G K G L E W V S G                                                        | <b>I S W N S G S I</b> | G Y A D S V K |
| <b>GCDS-L3</b> | G R F T I S R D N A N N S L F L Q M N S L R P E D T A L Y Y                                 |                        |               |
|                | GGCCGATTACCATCTCCAGAGACAACGCCAACAATTCCTGTTTCTGCAAATGAACAGTCTGCGACCTGAGGACACGGCCCTCTACTAC    |                        |               |
| <b>IGHV3-9</b> | .....G..C.....A.....A..G.....T.G..T...                                                      |                        |               |
|                | G R F T I S R D N A <u>K</u> N S L <u>Y</u> L Q M N S L R <u>A</u> E D T A L Y Y            |                        |               |

**FIGURE 3.** Cross-reactive IgG4 mAb genes are extensively mutated, especially in CDRs (boxed) compared with FWRs. The H-chain gene of a representative clone is aligned with its germline gene. Amino acids from germline sequence changed as a result of replacement mutations are underlined.

an individual before the onset of FS (40). To determine whether particular shared mutations are necessary for the development of the Dsg1/LJM11 specificity, the three shared VH genes (IGHV1-18, IGHV1-69, and IGHV4-34) from two FS patients (FGS and JLDO) were aligned to identify shared mutation among them. However, no shared mutation is present among all these VH gene-sharing mAbs between those from two patients (Fig. 4). Furthermore, the CDR3 sequences of these VH gene-sharing mAbs from two patients are also different, as shown in Table I. In general, H-chain CDR3 is important for Ab specificity (55, 56). Though our result suggests that the Dsg1/LJM11 specificity of cross-reactive Abs from FS may be governed by the combination of all three CDR sequences rather than by a single CDR such as CDR3. This suggests that the Ab response in different individuals is flexible and may have multiple routes to achieve the same specificity during the positive selection of their respective Ab evolution. The extensive mutations in FWRs among those FS autoantibodies (Table II) suggest that those mutations may also aid in improving the affinity of these autoantibodies to their target Ags in FS, as found previously in broadly neutralizing Abs to HIV-1 (57).

#### Germline VH and VL encoded revertant mAbs from FS recognize both Dsg1 and LJM11

The wide presence of Dsg1 and LJM11 cross-reactive Abs in FS suggests that either Dsg1 or LJM11 or both may participate in driving the development of these Abs. However, it does not reveal

whether Dsg1 or LJM11 could be the initial target for the naive B cells in FS. It is also possible that some other Ags (either environmental Ag or self-antigen), but not Dsg1 or LJM11, are the initial target that activates the naive B cells, which are the precursors for the eventual plasma cells that secrete these autoreactive Abs in FS. Therefore, it would be interesting to determine if naive B cells expressing germline V genes can bind Dsg1 and LJM11.

The mutations in the VH and VL genes of two cross-reactive mAbs, namely FGS-4E4 and GCDS-2D11 (their VH are in boldface in Table I), which were found using both the hybridoma method (13, 40) and APD method, were identified using IgBlast (45) and reverted back to the germline configurations (Fig. 5A). The DNA of the germline VH and VL genes of these two revertant mAbs were synthesized, subcloned into the original modified pComb3xSS vector (see *Materials and Methods*), produced, and purified as scFv (4E4-R and 2D11-R). The reactivity of these two revertant mAbs to Dsg1 and LJM11 was tested by ELISA and compared with that of their mutated counterpart scFv. As shown in Fig. 5B, these two revertant scFv react to both Dsg1 and LJM11. This suggests that either Dsg1 or LJM11 (or both) may be the initial target(s) of autoimmune responses in FS, further implicating the environmental contribution to the development of FS.

## Discussion

Our previous studies regarding the presence of cross-reactive mAbs in FS patients (13) suggest that there might be an overlap between

**Table II.** R/S in CDRs and FWRs of VH genes of the IgG4 scFv from FS patients

| Patient | VH                 | Total Clones | CDR            | FWR (R/S)      |
|---------|--------------------|--------------|----------------|----------------|
| GCDS    | <b>IGHV2-5*021</b> | <b>4</b>     | <b>12/4</b>    | <b>8/10</b>    |
|         | <b>IGHV3-9*01</b>  | <b>18</b>    | <b>108/18</b>  | <b>112/183</b> |
|         | IGHV3-53*04        | 2            | 12/6           | 36/16          |
|         | IGHV5-51*03        | 15           | 46/44          | 144/68         |
|         | <b>Total</b>       | <b>39</b>    | <b>178/72</b>  | <b>300/277</b> |
| FGS     | <b>IGHV1-18*01</b> | <b>4</b>     | <b>24/7</b>    | <b>42/24</b>   |
|         | <b>IGHV1-69*09</b> | <b>2</b>     | <b>6/0</b>     | <b>17/7</b>    |
|         | IGHV3-48*04        | 18           | 119/76         | 108/36         |
|         | <b>IGHV4-34*01</b> | <b>15</b>    | <b>33/0</b>    | <b>34/45</b>   |
|         | <b>IGHV4-59*08</b> | <b>6</b>     | <b>24/1</b>    | <b>59/34</b>   |
|         | IGHV7-4-1*02       | 13           | 39/24          | 49/119         |
|         | <b>Total</b>       | <b>58</b>    | <b>245/108</b> | <b>309/265</b> |
| JLDO    | <b>IGHV1-18*01</b> | <b>6</b>     | <b>12/0</b>    | <b>18/20</b>   |
|         | <b>IGHV1-69*09</b> | <b>7</b>     | <b>28/6</b>    | <b>34/5</b>    |
|         | <b>IGHV3-23*01</b> | <b>18</b>    | <b>126/18</b>  | <b>141/130</b> |
|         | <b>IGHV4-34*01</b> | <b>8</b>     | <b>32/1</b>    | <b>17/12</b>   |
|         | <b>Total</b>       | <b>39</b>    | <b>198/25</b>  | <b>210/167</b> |

The CDR R/S ratios of 10 clones are presented in bold.

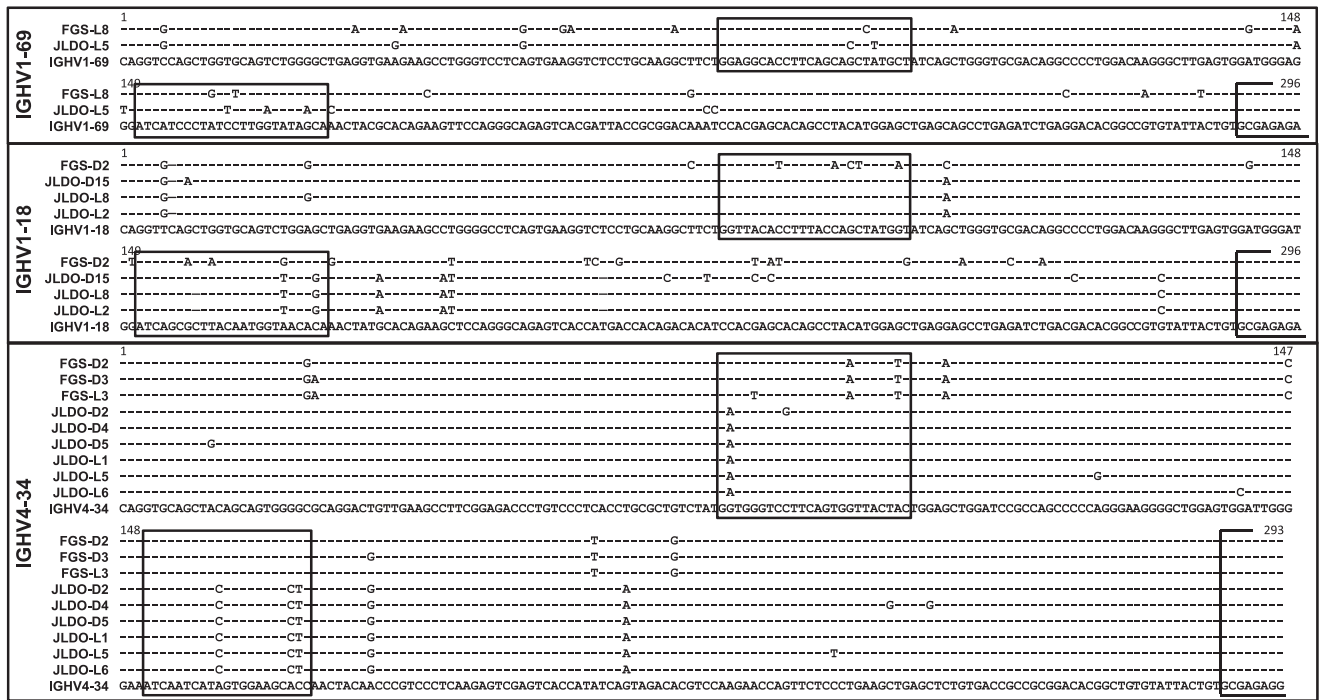


**FIGURE 4.** Alignments of IgG4 Ab clusters that use the same VH genes identified from two different patients. CDR1, CDR2, and partial CDR3 sequences are boxed. All nucleotide mutations are noted above the germline sequences.

the immune responses toward these two Ags. In this investigation, we seek to disclose the presence of an overlap between IgG4 responses against either Dsg1 or LJM11 in FS. Based on our hypothesis, the cross-reactive epitope(s) on Dsg1 and LJM11 would drive the development of cross-reactive Abs in FS. Due to the polyclonal nature of the Abs in patients' sera, the cross-reactivity of those IgG4 Abs can only be determined using monoclonal IgG4 Abs. The construction of IgG4-specific APD libraries and the subsequent panning of these libraries against either autoantigen or environmental Ag (i.e., LJM11 sand fly Ag) make it possible to identify these Ag-specific IgG4 Abs. Compared to the hybridoma method used in our previous investigation on FS Ab development (40), our newly developed IgG4 APD method modified from original APD method (41) has a distinct advantage over the hybridoma method. For example, only two IgG4 mAbs were isolated using hybridoma method and even several thousands of hybridoma clones were screened from eight FS patient B cell samples (40) (Y. Qian, unpublished observations). However, 14 IgG4 independent mAb clones are isolated from 3 FS patients using this IgG4 APD method. It indicates that IgG4 APD method has broader coverage of the Ab repertoire than hybridoma method. Moreover, this newly developed IgG4 APD method may have a wider application to the study of IgG4 Ab repertoires of other IgG4-related diseases in the future.

The presence of cross-reactive mAbs in FS (13) also suggests the presence of cross-reactive epitope(s) between autoantigen Dsg1 and environmental Ag LJM11. Notably, these two molecules are evolutionary distant and share little primary sequence homology. The cross-reactive mAbs from FS patients bind both molecules via conformational epitope (13), which does not rely on the similarity of their linear sequences. This explains the possible presence of a cross-reactive epitope on both molecules and also suggests that there are other epitopes on both molecules that are not cross-reactive, such as those epitopes that consist of continuous amino acids (linear epitopes). Thus, besides these Abs derived against the

cross-reactive epitope(s) on both molecules, it should be expected that there are Abs from FS patients that are either Dsg1- or LJM11-specific that do not cross-react to the other. It is quite surprising that all mAbs identified from our investigation are cross-reactive, regardless of whether they are obtained from panning against Dsg1 or LJM11. Even though it might be unlikely, we cannot rule out that these two molecules share more than one conformational epitope. Hence, it is possible that these mAbs bind different cross-reactive epitopes shared by these two molecules. Still, there might be other explanations. For example, the current screening method for mAb clones identified by panning of APD libraries only allows us to examine a limited number of clones. Thus, it is possible that only the most abundant clones were captured, whereas those rare clones, such as Dsg1- or LJM11-specific, escaped the detection. Nevertheless, the detection of identical and clonally related clones indicates that the most abundant IgG4 mAbs in these FS patients have been disclosed, and the presence of these cross-reactive IgG4 demonstrates the possible contribution of the environmental Ag in FS development. The rapid development of next generation sequencing method for high-throughput Ab sequence analysis will make it possible to identify those rare clones if the need arises in the future (58).

Another implication of our finding is that the cross-reactive epitope(s) might be the most immunogenic (or most dominant) that induces the strongest immune response against this epitope(s), as the conservation of the CDR mutations among most VH sharing mAbs identified from the same patient is remarkably stable. Thus, most IgG4 Abs from FS patients might be developed against this epitope. LJM11 is known to be the most immunogenic component in sand fly saliva (35), and it might be due to this cross-reactive epitope. In this regard, the cross-reactive epitope on Dsg1 must be a predominant epitope for the development of anti-Dsg1 Abs in FS. The presence of a predominant epitope is corroborated by other investigations on pemphigus. For instance, it has been shown that one predominant pathogenic epitope on Dsg1 may be targeted

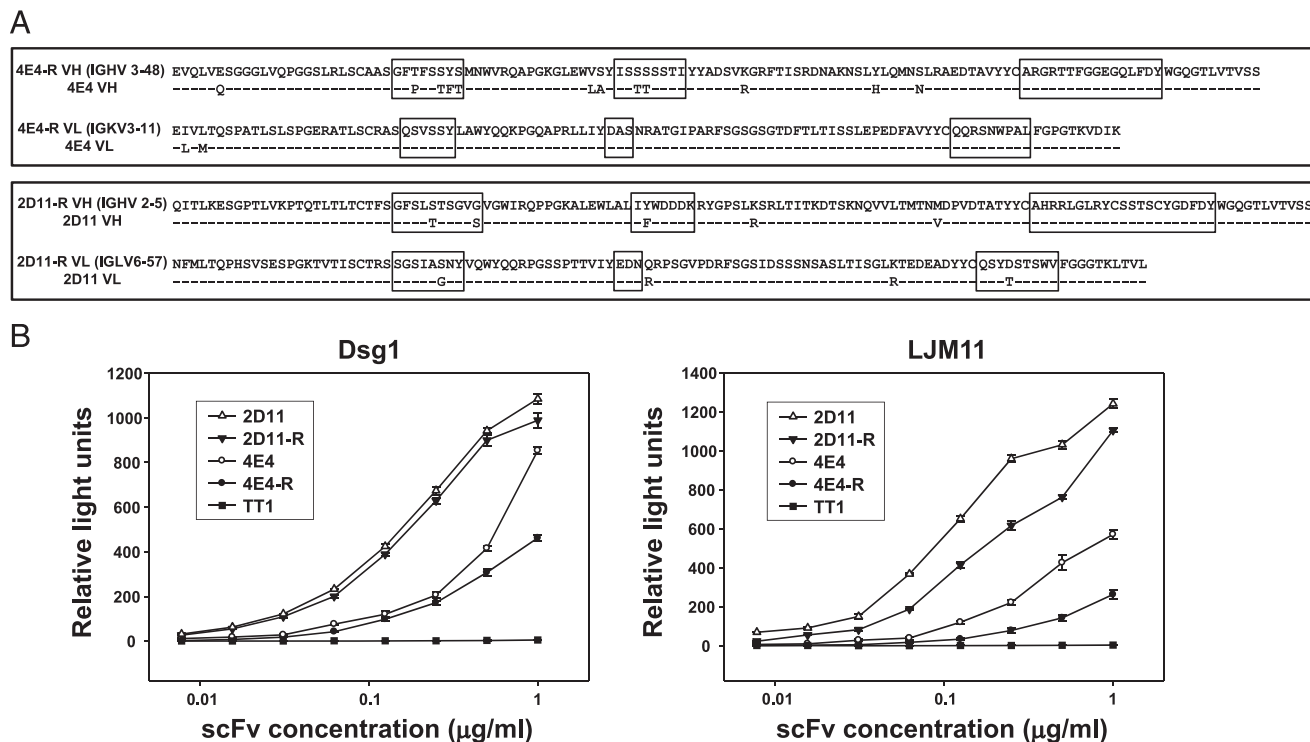


**FIGURE 5.** Revertant mAbs derived from two cross-reactive anti-Dsg1 mAbs from FS also recognize both Dsg1 and LJM11. **(A)** H- and L-chain amino acid sequences of 4E4 (*top panel*) and 2D11 (*bottom panel*) and their corresponding revertant counterparts (encoded by germline VH and VL genes). Only mutated amino acids are shown for the VH and VL chains of 4E4 and 2D11. The amino acid sequences of CDR1, -2, and -3 of VH and VL are boxed. **(B)** Similar to mutated anti-Dsg1 IgG4 scFv mAbs, their revertant scFv mAbs also react to both Dsg1 (*left panel*) and LJM11 (*right panel*). The negative control scFv (TT1) does not react with either Dsg1 or LJM11. The reactivity of each scFv Abs to either Dsg1 (*left panel*) or LJM11 (*right panel*) is measured by relative light units (RLU) as previously described (36). The results shown are representative of three independent experiments with triplicates in each experimental group.

by most PF patients, as the binding of an anti-Dsg1 pathogenic mAb can be inhibited by sera from these patients (48). Similarly, there is also an immunodominant epitope on Dsg3 in mucocutaneous pemphigus vulgaris (PV) (46). Not only is the binding of a pathogenic mAb from a PV patient to Dsg3 inhibited by these tested PV sera, but this pathogenic mAb also inhibits PV polyclonal Abs from binding to Dsg3 (46). Therefore, it is possible that all of these mAbs isolated from this study target one conformational epitope shared by Dsg1 and LJM11 or epitopes in the vicinity of or overlapping this epitope.

The isolated cross-reactive mAbs could be either pathogenic or not pathogenic. If these IgG4 autoantibodies are pathogenic, it would indicate that an environmental Ag may be directly involved in driving the pathogenic IgG4 autoantibody response in FS. If they are not pathogenic, it would suggest that these IgG4 autoantibodies may play a role in activating an extended autoantibody response, via mechanisms such as epitope spreading in FS, and this subsequent autoantibody response leads to the development of pathogenic autoantibodies in FS, as demonstrated before (59). In either case, the inciting Ag(s) (Dsg1 and/or LJM11) would be directly and/or indirectly involved in the development of pathogenic IgG4 autoantibodies in FS.

The current study also demonstrates that a noninfectious environmental Ag may contribute to the initiation of an autoimmune disease. It is possible, as shown in a previous study (36), that these FS genetically susceptible individuals mount an IgE response to sand fly Ag exposure. They then develop IgG4 Abs in response to the repeated (chronic) exposure to sand fly bites, because of the well-known transition from IgE to IgG4 during chronic antigenic stimulation or allergen-specific immunotherapy in allergy (60–

63). These IgG4 Abs turn out to be cross-reactive to Dsg1 autoantigen. Those anti-Dsg1 IgG4 Abs themselves or their triggered subsequent development of additional anti-Dsg1 IgG4 Abs result in the development of FS in those genetically susceptible individuals.

It is noteworthy that reverting mutated IgG4 autoantibodies from FS back to their germline VH and VL genes do not abolish their reactivity to both Dsg1 and LJM11. Numerous studies using revertant Abs to study human autoimmune diseases, such as pemphigus vulgaris and systemic lupus erythematosus indicate that the revertant Abs lose their reactivity (46, 64) or have reduced reactivity (65) to their corresponding autoantigens once their H- and L-chain V regions reverted back to their germline formats. Autoantibody development from non-autoreactive B cells has been proposed (64, 65). Due to the lack of candidate antigenic stimulants for triggering the development of these autoantibodies in human autoimmune diseases, the initial targets (except these autoantigens of their corresponding autoimmune diseases) for these naive B cells could not be further identified using revertant mAb method. In this study, we provide evidence that environmental Ags, such as LJM11 from sand fly, could be an antigenic stimulant that activates the naive B cells in those individuals who are genetically predisposed to FS disease and the subsequent development of IgG4 autoreactive Abs.

In summary, the extensive overlap of the IgG4 responses toward both Dsg1 autoantigen and LJM11 environmental Ag in this study is consistent with our hypothesis and thus establishes the possible link between the exposure to a noninfectious environmental Ag and the development of IgG4 autoantibodies in FS. Moreover, that revertant mAbs also cross-react to both

autoantigen (Dsg1) and environmental Ag (LJM11) provides further evidence that LJM11 could be the environmental Ag that triggers the activation of naive B cells. All of this evidence strongly suggests that LJM11 component from sand fly saliva as an environmental Ag may trigger the development of IgG4 autoantibodies in FS susceptible individuals in FS endemic regions. Our findings regarding the role of IgG4 response to an exogenous Ag in FS may be expanded to the understanding of the etiology of other IgG4-related autoimmune diseases.

## Disclosures

The authors have no financial conflicts of interest.

## References

- Diaz, L. A., S. A. Sampaio, E. A. Rivitti, C. R. Martins, P. R. Cunha, C. Lombardi, F. A. Almeida, R. M. Castro, M. L. Macca, C. Lavrado, et al. 1989. Endemic pemphigus foliaceus (Fogo Selvagem): II. Current and historic epidemiologic studies. *J. Invest. Dermatol.* 92: 4–12.
- Empinotti, J. C., L. A. Diaz, C. R. Martins, E. A. Rivitti, S. A. Sampaio, C. Lombardi, and J. A. Sanches, Cooperative Group for Fogo Selvagem Research. 1990. Endemic pemphigus foliaceus in western Parana, Brazil (1976–1988). *Br. J. Dermatol.* 123: 431–437.
- Lever, W. F. 1953. Pemphigus. *Medicine (Baltimore)* 32: 1–123.
- Beutner, E. H., and R. E. Jordan. 1964. Demonstration of Skin Antibodies in Sera of Pemphigus Vulgaris Patients by Indirect Immunofluorescent Staining. *Proc. Soc. Exp. Biol. Med.* 117: 505–510.
- Udey, M. C., and J. R. Stanley. 1999. Pemphigus—diseases of antidesmosomal autoimmunity. *JAMA* 282: 572–576.
- Anhalt, G. J., and L. A. Diaz. 2001. Prospects for autoimmune disease: Research advances in pemphigus. *JAMA* 285: 652–654.
- Amagai, M. 2003. Desmoglein as a target in autoimmunity and infection. *J. Am. Acad. Dermatol.* 48: 244–252.
- Ding, X., V. Aoki, J. M. Mascaro, Jr., A. Lopez-Swiderski, L. A. Diaz, and J. A. Fairley. 1997. Mucosal and mucocutaneous (generalized) pemphigus vulgaris show distinct autoantibody profiles. *J. Invest. Dermatol.* 109: 592–596.
- Diaz, L. A., S. A. Sampaio, E. A. Rivitti, C. R. Martins, P. R. Cunha, C. Lombardi, F. A. Almeida, R. M. Castro, M. L. Macca, C. Lavrado, et al. 1989. Endemic pemphigus foliaceus (fogo selvagem). I. Clinical features and immunopathology. *J. Am. Acad. Dermatol.* 20: 657–669.
- Stanley, J. R., V. Klaus-Kovtun, and S. A. Sampaio. 1986. Antigenic specificity of fogo selvagem autoantibodies is similar to North American pemphigus foliaceus and distinct from pemphigus vulgaris autoantibodies. *J. Invest. Dermatol.* 87: 197–201.
- Moraes, M. E., M. Fernandez-Vina, A. Lazaro, L. A. Diaz, G. H. Filho, H. Friedman, E. Rivitti, V. Aoki, P. Stastny, and J. R. Moraes. 1997. An epitope in the third hypervariable region of the DRB1 gene is involved in the susceptibility to endemic pemphigus foliaceus (fogo selvagem) in three different Brazilian populations. *Tissue Antigens* 49: 35–40.
- Aoki, V., R. C. Millikan, E. A. Rivitti, G. Hans-Filho, D. P. Eaton, S. J. Warren, N. Li, J. Hilario-Vargas, R. G. Hoffmann, and L. A. Diaz, Cooperative Group for Fogo Selvagem Research. 2004. Environmental risk factors in endemic pemphigus foliaceus (fogo selvagem). *J. Invest. Dermatol. Symp. Proc.* 9: 34–40.
- Qian, Y., J. S. Jeong, M. Maldonado, J. G. Valenzuela, R. Gomes, C. Teixeira, F. Evangelista, B. Qaqish, V. Aoki, G. Hans, Jr., et al. 2012. Cutting Edge: Brazilian pemphigus foliaceus anti-desmoglein 1 autoantibodies cross-react with sand fly salivary LJM11 antigen. *J. Immunol.* 189: 1535–1539.
- Goodnow, C. C. 2007. Multistep pathogenesis of autoimmune disease. *Cell* 130: 25–35.
- Fierabracci, A. 2009. Unravelling the role of infectious agents in the pathogenesis of human autoimmunity: the hypothesis of the retroviral involvement revisited. *Curr. Mol. Med.* 9: 1024–1033.
- Chervonsky, A. V. 2010. Influence of microbial environment on autoimmunity. *Nat. Immunol.* 11: 28–35.
- Miller, F. W. 2011. Environmental agents and autoimmune diseases. *Adv. Exp. Med. Biol.* 711: 61–81.
- Miller, F. W., L. Alfredsson, K. H. Costenbader, D. L. Kamen, L. M. Nelson, J. M. Norris, and A. J. De Roos. 2012. Epidemiology of environmental exposures and human autoimmune diseases: findings from a National Institute of Environmental Health Sciences Expert Panel Workshop. *J. Autoimmun.* 39: 259–271.
- Germolec, D., D. H. Kono, J. C. Pfau, and K. M. Pollard. 2012. Animal models used to examine the role of the environment in the development of autoimmune disease: findings from an NIEHS Expert Panel Workshop. *J. Autoimmun.* 39: 285–293.
- Rock, B., C. R. Martins, A. N. Theofilopoulos, R. S. Balderas, G. J. Anhalt, R. S. Labib, S. Futamura, E. A. Rivitti, and L. A. Diaz. 1989. The pathogenic effect of IgG4 autoantibodies in endemic pemphigus foliaceus (fogo selvagem). *N. Engl. J. Med.* 320: 1463–1469.
- Warren, S. J., L. A. Arteaga, E. A. Rivitti, V. Aoki, G. Hans-Filho, B. F. Qaqish, M. S. Lin, G. J. Giudice, and L. A. Diaz. 2003. The role of subclass switching in the pathogenesis of endemic pemphigus foliaceus. *J. Invest. Dermatol.* 120: 104–108.
- Qaqish, B. F., P. Prisanh, Y. Qian, E. Andraca, N. Li, V. Aoki, G. Hans-Filho, V. dos Santos, E. A. Rivitti, and L. A. Diaz, Cooperative Group on Fogo Selvagem Research. 2009. Development of an IgG4-based predictor of endemic pemphigus foliaceus (fogo selvagem). *J. Invest. Dermatol.* 129: 110–118.
- Sitaru, C., S. Mihai, and D. Zillikens. 2007. The relevance of the IgG subclass of autoantibodies for blister induction in autoimmune bullous skin diseases. *Arch. Dermatol. Res.* 299: 1–8.
- Kamisawa, T., and A. Okamoto. 2006. Autoimmune pancreatitis: proposal of IgG4-related sclerosing disease. *J. Gastroenterol.* 41: 613–625.
- Yamamoto, M., H. Takahashi, S. Sugai, and K. Imai. 2005. Clinical and pathological characteristics of Mikulicz's disease (IgG4-related plasmacytic exocrinopathy). *Autoimmun. Rev.* 4: 195–200.
- Stone, J. H., Y. Zen, and V. Deshpande. 2012. IgG4-related disease. *N. Engl. J. Med.* 366: 539–551.
- Stone, J. H., A. Khosroshahi, V. Deshpande, J. K. Chan, J. G. Heathcote, R. Aalberse, A. Azumi, D. B. Bloch, W. R. Brugge, M. N. Carruthers, et al. 2012. Recommendations for the nomenclature of IgG4-related disease and its individual organ system manifestations. *Arthritis Rheum.* 64: 3061–3067.
- Tokura, Y., H. Yagi, H. Yanaguchi, Y. Majima, A. Kasuya, T. Ito, M. Maekawa, and H. Hashizume. 2014. IgG4-related skin disease. *Br. J. Dermatol.* 171: 959–967.
- Roscoe, J. T., L. Diaz, S. A. Sampaio, R. M. Castro, R. S. Labib, Y. Takahashi, H. Patel, and G. J. Anhalt. 1985. Brazilian pemphigus foliaceus autoantibodies are pathogenic to BALB/c mice by passive transfer. *J. Invest. Dermatol.* 85: 538–541.
- Anhalt, G. J., R. S. Labib, J. J. Voorhees, T. F. Beals, and L. A. Diaz. 1982. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. *N. Engl. J. Med.* 306: 1189–1196.
- Gourley, M., and F. W. Miller. 2007. Mechanisms of disease: Environmental factors in the pathogenesis of rheumatic disease. *Nat. Clin. Pract. Rheumatol.* 3: 172–180.
- Cooper, G. S., K. M. Gilbert, E. L. Greidinger, J. A. James, J. C. Pfau, L. Reinlib, B. C. Richardson, and N. R. Rose. 2008. Recent advances and opportunities in research on lupus: environmental influences and mechanisms of disease. *Environ. Health Perspect.* 116: 695–702.
- Münz, C., J. D. Lünemann, M. T. Getts, and S. D. Miller. 2009. Antiviral immune responses: triggers of or triggered by autoimmunity? *Nat. Rev. Immunol.* 9: 246–258.
- Francis, L., and A. Perl. 2010. Infection in systemic lupus erythematosus: friend or foe? *Int. J. Clin. Rheumatol.* 5: 59–74.
- Teixeira, C., R. Gomes, N. Collin, D. Reynoso, R. Jochim, F. Oliveira, A. Seitz, D. E. Elnaïem, A. Caldas, A. P. de Souza, et al. 2010. Discovery of markers of exposure specific to bites of *Lutzomyia longipalpis*, the vector of *Leishmania infantum* chagasi in Latin America. *PLoS Negl. Trop. Dis.* 4: e638.
- Qian, Y., J. S. Jeong, M. Abdeladhim, J. G. Valenzuela, V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz, Cooperative Group on Fogo Selvagem Research. 2015. IgE anti-LJM11 sand fly salivary antigen may herald the onset of fogo selvagem in endemic Brazilian regions. *J. Invest. Dermatol.* 135: 913–915.
- McClain, M. T., L. D. Heinlen, G. J. Dennis, J. Roebuck, J. B. Harley, and J. A. James. 2005. Early events in lupus humoral autoimmunity suggest initiation through molecular mimicry. *Nat. Med.* 11: 85–89.
- Cusick, M. F., J. E. Libbey, and R. S. Fujinami. 2012. Molecular mimicry as a mechanism of autoimmune disease. *Clin. Rev. Allergy Immunol.* 42: 102–111.
- Joseph, C. G., E. Darrah, A. A. Shah, A. D. Skora, L. A. Casciola-Rosen, F. M. Wigley, F. Boin, A. Fava, C. Thoburn, I. Kinde, et al. 2014. Association of the autoimmune disease scleroderma with an immunologic response to cancer. *Science* 343: 152–157.
- Qian, Y., S. H. Clarke, V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz. 2009. Antigen selection of anti-DSG1 autoantibodies during and before the onset of endemic pemphigus foliaceus. *J. Invest. Dermatol.* 129: 2823–2834.
- Barbas, C. F. I., D. R. Burton, J. K. Scott, and G. J. Silverman. 2001. *Phage display: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Efremov, D. G., F. D. Batista, and O. R. Burrone. 1993. Molecular analysis of IgE H-chain transcripts expressed in vivo by peripheral blood lymphocytes from normal and atopic individuals. *J. Immunol.* 151: 2195–2207.
- Xu, X., F. Oliveira, B. W. Chang, N. Collin, R. Gomes, C. Teixeira, D. Reynoso, V. My Pham, D. E. Elnaïem, S. Kamhawi, et al. 2011. Structure and function of a “yellow” protein from saliva of the sand fly *Lutzomyia longipalpis* that confers protective immunity against *Leishmania major* infection. *J. Biol. Chem.* 286: 32383–32393.
- Qian, Y., P. Prisanh, E. Andraca, B. F. Qaqish, V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz, Cooperative Group on Fogo Selvagem Research. 2011. IgE, IgM, and IgG4 anti-desmoglein 1 autoantibody profile in endemic pemphigus foliaceus (fogo selvagem). *J. Invest. Dermatol.* 131: 985–987.
- Ye, J., N. Ma, T. L. Madden, and J. M. Ostell. 2013. IgBLAST: an immunoglobulin variable domain sequence analysis tool. *Nucleic Acids Res.* 41: W34–W40.
- Di Zenzo, G., G. Di Lullo, D. Corti, V. Calabresi, A. Sinistro, F. Vanzetta, B. Didona, G. Cianchini, M. Herti, R. Emig, et al. 2012. Pemphigus autoantibodies generated through somatic mutations target the desmoglein-3 cis-interface. *J. Clin. Invest.* 122: 3781–3790.
- Payne, A. S., K. Ishii, S. Kacir, C. Lin, H. Li, Y. Hanakawa, K. Tsunoda, M. Amagai, J. R. Stanley, and D. L. Siegel. 2005. Genetic and functional characterization of human pemphigus vulgaris monoclonal autoantibodies isolated by phage display. *J. Clin. Invest.* 115: 888–899.
- Ishii, K., C. Lin, D. L. Siegel, and J. R. Stanley. 2008. Isolation of pathogenic monoclonal anti-desmoglein 1 human antibodies by phage display of pemphigus foliaceus autoantibodies. *J. Invest. Dermatol.* 128: 939–948.

49. Yamagami, J., S. Kacir, K. Ishii, A. S. Payne, D. L. Siegel, and J. R. Stanley. 2009. Antibodies to the desmoglein 1 precursor proprotein but not to the mature cell surface protein cloned from individuals without pemphigus. *J. Immunol.* 183: 5615–5621.
50. Yamagami, J., A. S. Payne, S. Kacir, K. Ishii, D. L. Siegel, and J. R. Stanley. 2010. Homologous regions of autoantibody heavy chain complementarity-determining region 3 (H-CDR3) in patients with pemphigus cause pathogenicity. *J. Clin. Invest.* 120: 4111–4117.
51. Snow, R. E., C. J. Chapman, S. T. Holgate, and F. K. Stevenson. 1998. Clonally related IgE and IgG4 transcripts in blood lymphocytes of patients with asthma reveal differing patterns of somatic mutation. *Eur. J. Immunol.* 28: 3354–3361.
52. Link, J. M., and H. W. Schroeder, Jr. 2002. Clues to the etiology of autoimmune diseases through analysis of immunoglobulin genes. *Arthritis Res.* 4: 80–83.
53. Foreman, A. L., J. Van de Water, M. L. Gougeon, and M. E. Gershwin. 2007. B cells in autoimmune diseases: insights from analyses of immunoglobulin variable (Ig V) gene usage. *Autoimmun. Rev.* 6: 387–401.
54. Shlomchik, M. J., A. Marshak-Rothstein, C. B. Wolfowicz, T. L. Rothstein, and M. G. Weigert. 1987. The role of clonal selection and somatic mutation in autoimmunity. *Nature* 328: 805–811.
55. Mahmoud, T. I., H. W. Schroeder, Jr., and J. F. Kearney. 2011. Limiting CDR-H3 diversity abrogates the antibody response to the bacterial polysaccharide  $\alpha$  1 $\rightarrow$ 3 dextran. *J. Immunol.* 187: 879–886.
56. Silva-Sanchez, A., C. R. Liu, A. M. Vale, M. Khass, P. Kapoor, A. Elgavish, I. I. Ivanov, G. C. Ippolito, R. L. Schelonka, T. R. Schoeb, et al. 2015. Violation of an evolutionarily conserved immunoglobulin diversity gene sequence preference promotes production of dsDNA-specific IgG antibodies. *PLoS One* 10: e0118171.
57. Klein, F., R. Diskin, J. F. Scheid, C. Gaebler, H. Mouquet, I. S. Georgiev, M. Pancera, T. Zhou, R. B. Incesu, B. Z. Fu, et al. 2013. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell* 153: 126–138.
58. Ravn, U., G. Didelot, S. Venet, K. T. Ng, F. Gueneau, F. Rousseau, S. Calloud, M. Kosco-Vilbois, and N. Fischer. 2013. Deep sequencing of phage display libraries to support antibody discovery. *Methods* 60: 99–110.
59. Li, N., V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz. 2003. The role of intramolecular epitope spreading in the pathogenesis of endemic pemphigus foliaceus (fogo selvagem). *J. Exp. Med.* 197: 1501–1510.
60. Aalberse, R. C., R. van der Gaag, and J. van Leeuwen. 1983. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J. Immunol.* 130: 722–726.
61. Gleich, G. J., E. M. Zimmermann, L. L. Henderson, and J. W. Yunginger. 1982. Effect of immunotherapy on immunoglobulin E and immunoglobulin G antibodies to ragweed antigens: a six-year prospective study. *J. Allergy Clin. Immunol.* 70: 261–271.
62. Rossi, R. E., G. Monasterolo, G. Coco, L. Silvestro, and D. Operti. 2007. Evaluation of serum IgG4 antibodies specific to grass pollen allergen components in the follow up of allergic patients undergoing subcutaneous and sublingual immunotherapy. *Vaccine* 25: 957–964.
63. Golden, D. B., I. D. Lawrence, R. H. Hamilton, A. Kagey-Sobotka, M. D. Valentine, and L. M. Lichtenstein. 1992. Clinical correlation of the venom-specific IgG antibody level during maintenance venom immunotherapy. *J. Allergy Clin. Immunol.* 90: 386–393.
64. Wellmann, U., M. Letz, M. Herrmann, S. Angermüller, J. R. Kalden, and T. H. Winkler. 2005. The evolution of human anti-double-stranded DNA autoantibodies. *Proc. Natl. Acad. Sci. USA* 102: 9258–9263.
65. Zhang, J., A. M. Jacobi, T. Wang, and B. Diamond. 2008. Pathogenic autoantibodies in systemic lupus erythematosus are derived from both self-reactive and non-self-reactive B cells. *Mol. Med.* 14: 675–681.



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# Overlapping IgG4 Responses to Self- and Environmental Antigens in Endemic Pemphigus Foliaceus

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The etiology of human autoimmune diseases in general remains largely unknown, although the genetic and environmental interplay may be relevant. This applies to the autoimmune diseases of the skin such as the pemphigus phenotypes and others. In this group, there is an endemic form of pemphigus foliaceus (also known as fogo selvagem [FS]) in which the pathogenic IgG4 autoantibody response to the self-antigen desmoglein 1 (Dsg1) cross-reacts with the LJM11 sand fly salivary gland Ag. In this investigation, we dissected the IgG4 autoantibody repertoires used by FS patients in response to endogenous self-Dsg1 and exogenous LJM11 sand fly Ag. Based on analyses of the genetic clonal signatures of these Abs, our results indicate that there is a significant overlap between these two responses, as all identified IgG4 mAbs cross-react to both Dsg1 and LJM11 Ags. Germline H- and L-chain V gene Abs generated according to mutated cross-reactive mAbs preserved their reactivity to both Ags. Our findings suggest that both Dsg1 autoantigen and LJM11 environmental Ag could be the initial antigenic stimulants for the IgG4 autoimmune responses in FS. These results support our hypothesis that LJM11 Ag plays a substantial role in triggering the IgG4 autoantibody development in FS and provide new insights on how noninfectious environmental Ag(s) may drive the generation of autoantibodies in IgG4-related autoimmune diseases. *The Journal of Immunology*, 2016, 196: 2041–2050.

**F**ogo selvagem (FS) is an endemic form of pemphigus foliaceus (PF) found in certain states of Brazil (1, 2). The hallmark of this disease is the presence of intraepidermal vesicles due to epidermal cell detachment (acantholysis) (3) induced by pathogenic IgG4 anti-desmoglein 1 (Dsg1) autoantibodies (4–8). FS shows similar clinical, histological, and immunological features to those observed in nonendemic PF (9, 10). Epidemiological and immunogenetic studies suggest that both genetic and environmental factors contribute to the development of FS (1, 11, 12). Previous studies suggest that exposure to hematophagous insect bites in genetically predisposed individuals may be a risk factor for FS (12). To strengthen this hypothesis, we have shown that IgG4 anti-Dsg1 autoantibodies cross-react with LJM11 sand fly salivary gland Ag (13), which suggests that the development of IgG4 Abs may be linked to immune responses to environmental Ags. Com-

pared to investigations on the pathogenesis and genetic predisposition of autoimmune diseases, etiological studies regarding environmental triggers of these diseases are lacking due to low prevalence and the clinical heterogeneity of the diseases (14–19). Similarly, the random nature of autoimmune skin diseases in North America makes it difficult to assess their etiological commonality and further dissect their causes. In this regard, the endemic nature of FS provides an invaluable model and rare opportunity to study the environmental factors within the FS endemic region and their contribution to the development of FS.

IgG4 Abs are known to be elevated in patients with FS (20–22) and other bullous dermatoses (23), as well as autoimmune pancreatitis (24), Mikulicz's disease (primary Sjögren's syndrome) (25), and other diseases (26). Recently, the terms IgG4-related disease and IgG4-related skin disease have been proposed (26–28). Among some autoimmune diseases, increased serum levels of total IgG4 are often observed, and certain specific histopathological features, such as IgG4 plasma cell infiltration in effected tissues or organs, are present (26–28). In contrast, increased circulating anti-Dsg1 IgG4 autoantibodies are characteristic of FS/PF, as these anti-epidermal autoantibodies are pathogenic and detected bound to the surface of detached keratinocytes in lesional and perilesional epidermis of these patients (4). In FS/PF, the lesional skin does not show IgG4 B cell or plasma cell infiltrates. In 1989, Rock et al. (20) demonstrated that the IgG4 response in FS is pathogenic. Later studies confirmed that the bulk of pathogenic anti-Dsg1 autoantibodies in FS are predominantly IgG4 (21). IgG4 anti-Dsg1 Abs from FS patients are pathogenic in mice (8, 29), similar to those in PF (30) using an IgG passive transfer mouse model. One study showed that progression from the pre-clinical to the clinical stage of the disease is associated with a dramatic rise in IgG4 anti-Dsg1 autoantibodies (21) and that the level of anti-Dsg1 IgG4 Abs can be used as a predictor of FS (22). Our recent finding that IgG4 autoantibodies in FS cross-react with an LJM11 sand fly Ag (13) suggests that the development of IgG4 autoantibodies in patients may be linked to exposure to an envi-

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Abbreviations used in this article: APD, Ab phage display; Dsg1, desmoglein 1; FS, fogo selvagem; FWR, framework region; PF, pemphigus foliaceus; PV, pemphigus vulgaris; R/S, replacement and silent mutations; scFv, single-chain fragment variable.

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ronmental Ag. The studies of the association of environmental factors, such as infectious agents, with the development of autoimmune diseases have a long history (15, 17–19, 31–34). However, the studies of noninfectious Ags and their association with the development of autoimmune diseases are scarce. Because sand flies are prevalent in FS endemic areas, it is possible that individuals living in these endemic regions, especially among those FS-susceptible individuals, may generate an immune response to Ags from sand fly saliva (i.e., LJM11). The LJM11 Ag, a member of the yellow family of salivary proteins, is one of the most immunogenic component among the sand fly saliva proteins and also a marker for human exposure of sand fly bites (35). That anti-LJM11 Abs cross-react to autoantigen Dsg1 (13, 36) strongly suggests the existence of a cross-reactive epitope(s) on both LJM11 and Dsg1. Because exposure to exogenous Ags may induce the autoimmune response to cross-reactive autoantigens in autoimmune diseases (33, 34, 37–39), we hypothesize that the immune response directed against this cross-reactive epitope(s) subsequently initiates the generation of IgG4 autoantibodies in FS-susceptible individuals.

The aim of this investigation was to determine if the IgG4 autoantibody responses to Dsg1 and LJM11 sand fly Ags overlap at the Ab V gene level on FS patients from endemic areas. Our study indicates that there is a striking overlap between these two IgG4 responses and LJM11 could be an inciting Ag for autoantibody development, strongly suggesting that LJM11 sand fly Ag plays an important role in the etiology of FS.

## Materials and Methods

### *Immortalized B cell samples from FS patients*

Immortalized B cell samples from FS patients in this investigation have been described before (40) and kept at the University of North Carolina Dermatology Research Laboratories. These studies are approved by the institutional review boards from the University of North Carolina at Chapel Hill.

### *IgG4 Ab phage display library construction*

To amplify the VH and VL genes for Ab phage display (APD) library construction, mRNA from the immortalized B cells of three FS patients (13) were isolated, and their cDNA were synthesized. The L-chain V genes were amplified as originally described (41). To obtain IgG4 only H-chain genes, we used the 5' primers described from the original method (41), whereas the 3' primers for VH genes from the original method (41) were replaced by an IgG4-specific primer based on an IgG4 Ab C-region-specific sequence designed by Efremov et al. (42). A 209-bp DNA segment from the H-chain C-region (including 36 bp from the IgG4 hinge region) was amplified along with the H-chain V genes transcripts. The resulting IgG4 single-chain fragment variable (scFv) comprises L- and H-chain V segments and partial C-region of IgG4 H-chain (Supplemental Fig. 1). The subsequent APD library construction was according to the original method described (41). To introduce FLAG-tag at the N-terminal of the resulting scFv for more convenient detection and purification, the original pComb3xSS vector was modified. The primer sequences used for PCR are: 5'–ATAGGCCAGGCCGCGCCAGCACCATCACCATCACCATTAGGAG–GGTGGTGGCTCTG–3' (for removing HA at C-terminal) and 5'–ATAGGCCGCTGGGCCAACTTGTCATCGTCATCCTTGTAATCCGCA–GCTTGGGCCACGGTAGCGAA–3' (for adding FLAG at N-terminal). PCR cycle consisted of: 94°C, 4 min for one cycle; 94°C for 30 s/55°C for 30 s/72°C for 5 min for 30 cycles; and 72°C for 10 min for one cycle. This PCR product was SfiI digested and cloned to modified pComb3xSS vector (with FLAG-tag at the N-terminal).

### *Isolation of anti-Dsg1, anti-LJM11, and cross-reactive mAbs*

The constructed IgG4 APD libraries were panned as described (41) against either Dsg1 or LJM11 for three rounds or the alternation of Dsg1 and LJM11 for total of four rounds (Supplemental Table I). These selected clones were amplified and their H- and L-chain sequenced. Selected clones were subjected to scFv protein expression and production. Produced scFv mAbs were purified by either Nickel column (Qiagen) or anti-FLAG magnetic beads (Sigma-Aldrich, St. Louis, MO) according to the manufacturers' instruction for further testing. Human Dsg1 and sand fly LJM11 protein were purified as described (13, 35, 36, 40, 43).

### *ELISA and other mAbs*

The specificity of those purified scFv mAbs were tested by ELISA as previous described (13, 36, 40, 44). Negative control scFv (TT1) was kindly provided by Drs. Don Siegel and Stephen Kacir from the University of Pennsylvania and subcloned in our modified pComb3xSS vector with an N-terminal FLAG-tag. Anti-FLAG-HRP (Sigma-Aldrich) was used as secondary Ab for detection of all purified scFv mAbs.

### *Revertant scFv mAbs*

The germline sequences of the H- and L-chain of two mAbs (4E4 and 2D11) isolated previously (40) were identified using The National Center for Biotechnology Information (IgBlast) (45). The H- and L-chain V genes of the germline formats of these two mAbs were synthesized (GenScript, Piscataway, NJ) and subcloned into our modified pComb3xSS vector. The revertant scFv mAbs (4E4-R and 2D11-R) and their mutated counterpart scFv mAbs (4E4 and 2D11) were expressed and purified as described (41).

### *VH gene analysis*

The identified scFv mAb H-chain genes were analyzed using Ab gene database at The National Center for Biotechnology Information (IgBlast) (45). Vector NTI (Invitrogen) is used for multiple alignments of the VH genes.

### *Statistics*

The significant difference between the ratio of replacement and silent mutations (R/S) for CDRs and framework regions (FWRs) is assessed using  $\chi^2$  test.

## Results

### *Construction of IgG4 APD libraries from FS B cells*

Because the main pathogenic autoantibodies are of the IgG4 subclass in FS (20, 21), we sought to focus on the characterization of IgG4 Abs from FS patients. The APD library constructed using the method originally described by Barbas et al. (41) includes the Ab repertoire from all four IgG subclasses in a constructed IgG phage display library, and the original IgG subclass identity could not be determined from resulting scFv Abs from the library. However, because of the predominant presence of IgG4 Abs in autoimmune skin diseases (4–8) and other IgG4-related diseases (26), the identification and isolation of IgG4 mAbs from these patients became increasingly necessary to understand the mechanism of IgG4 Ab development in these patients. Thus, we have modified the method for our particular purpose to obtain only IgG4 mAbs (Supplemental Fig. 1). The advantages of the resulting mAbs are that: 1) the scFv Abs in these libraries derived from FS B cells comprise IgG4 Abs only; and 2) the scFv contain partial constant and hinge regions of IgG4 immediately following VH domain, and their IgG4 subclass identity can be further confirmed. Employing this strategy, we have constructed three IgG4 phage display libraries using mRNA isolated from three FS patients' EBV-immortalized B cells.

### *Isolation of anti-Dsg1 and anti-LJM11 IgG4 mAbs by panning against Dsg1 and LJM11*

We have shown that FS patients have significantly higher IgG4 anti-Dsg1 and LJM11 Abs compared with normal controls from both FS (Lima Verde in Brazil) and non-FS endemic region (36), suggesting that there are IgG4 immune responses against both the autoantigen and environmental Ag. Additionally, that two anti-Dsg1 IgG4 mAbs isolated from FS patients previously using hybridoma method cross-react to both Dsg1 and LJM11 (13) suggests the presence of cross-reactive epitope(s). Thus, it is possible that IgG4 Abs are present in FS patients reacting to both Dsg1 and LJM11.

To determine whether there is an overlap between these anti-Dsg1 and anti-LJM11 IgG4 responses, we identified the anti-Dsg1 and anti-LJM11 IgG4 Abs from the IgG4 APD libraries

constructed from B cells of 3 FS patients to study the IgG4 Ab repertoires by panning against either Dsg1 and/or LJM11 (Supplemental Table I) (41). Colonies were randomly selected from Dsg1 and LJM11 panning of these three IgG4 APD libraries. Their plasmids were purified and subject to sequencing using Ompseq and HRML-F primers as described (41). The Ab H- and L-chain gene sequencing results of selected Ab clones from IgG4 APD libraries were analyzed using Ab gene database at The National Center for Biotechnology Information (IgBlast) (45) and their four FWRs and three CDRs determined. Totals of 39 (GCDS), 58 (FGS), and 39 (JLDO) clones from Dsg1 and LJM11 panning were successfully sequenced, and their VH and VL gene usages were determined. The additional H-chain C-region sequences of these mAbs were also analyzed and confirmed to be of IgG4 subclass, indicating that only IgG4 mAbs were identified from our constructed IgG4 APD libraries.

*Same parental B cell origins by IgG4 mAb clones isolated from panning of IgG4 APD libraries against either Dsg1 autoantigen or LJM11 environmental Ag*

The H-chain V regions of mAbs from pemphigus are known to contribute most of the Dsg binding. For example, Di Zenzo et al. (46) demonstrated that H-chain V gene somatic mutations are required for mAbs to maintain its binding to Dsg3, whereas L-chain V gene mutations are not required for their binding to Dsg3. Studies from the Stanley group indicated that the H-chain of an anti-Dsg mAb could pair with L-chains encoded by different VL genes without losing its Dsg specificity (47–50). Consistent with all of these previous findings, we also found that most of independent mAbs identified from either Dsg1 or LJM11 panning of FS IgG4 APD libraries have different L-chains paired with the same H-chains (data not shown). Therefore, all of our genetic analyses of these

mAbs in this investigation are centered on the H-chain V gene segments. Among all three CDRs, the CDR3 region is the most diverse part of the Ab V regions, and a unique CDR3 sequence represents the genetic clonal signature for a group of Abs derived from the same parental B cell. Based on their H-chain V gene usage and the clonal signature of clonally related Abs (51), these clones can be grouped to four, six, and four independent clones from three FS patients, respectively (Table I). It should be noted that the VH genes of two IgG4 mAbs (GCDS-2D11 and FGS-4E4) that were isolated using hybridoma method from our previous study (40) are also identified from these clones isolated in our current study. The H-chain of GCDS-2D11 is encoded by IGHV2-5, whereas that of FGS-4E4 is encoded by IGHV3-48. Both clusters of mAbs encoded by these two VH genes and their respective CDR3s, are identified from their corresponding IgG4 APD libraries (both VH genes are in boldface in Table I). It indicates that Ag-specific mAbs can be identified using both hybridoma and APD libraries, further validating our newly developed method for construction and panning of IgG4 APD libraries to identify only IgG4 mAbs.

We next used two methods to further determine whether these mAbs are cross-reactive to Dsg1 and LJM11. These three IgG4 APD libraries were panned against the alternation of Dsg1 and LJM11 for total four rounds (panned against Dsg1 for first and third rounds and panned against LJM11 for second and fourth rounds) (Supplemental Table I) to identify cross-reactive mAbs. Those identified clones were sequenced and compared with those clones identified from Dsg1 or LJM11 individual panning. Most mAbs found from Dsg1- and LJM11-only panning are also found from the Dsg1/LJM11 alternate panning and thus determined to be cross-reactive. Some representative scFv were produced and purified as described (41) and tested by both anti-Dsg1 and LJM11

Table I. IgG4 scFv isolated from panning of IgG4 APD libraries

| Patient | VH                             | CDR3 (FWR4)                                                   | Clones Identified from Panning |       | Cross-Reactivity Confirmed by         |
|---------|--------------------------------|---------------------------------------------------------------|--------------------------------|-------|---------------------------------------|
|         |                                |                                                               | Dsg1                           | LJM11 |                                       |
| GCDS    | <b>IGHV2-5*02<sup>a</sup></b>  | AHRRRLGLRYCSTSSCFG <b>DFDY<sup>b</sup></b><br>(WGQGTTLTVSS)   | 3                              | 1     | ELISA <sup>d</sup>                    |
|         | IGHV3-9*01                     | VKSDSTGSSIRDASIFDS<br>(WGQGSLLTVSS)                           | 9                              | 9     | Alternate panning, <sup>e</sup> ELISA |
|         | IGHV3-53*04                    | ARSLLSVGRYFDL(WGRGTLTVSS)                                     | 0 <sup>c</sup>                 | 2     | ELISA                                 |
|         | IGHV5-51*03                    | ARSYNSSE <b>YYIWFEA</b> (WGQGTTLTVSS) <sup>b</sup>            | 11                             | 4     | Alternate panning, ELISA              |
| Total   |                                |                                                               | 23                             | 16    |                                       |
| FGS     | IGHV1-18*01                    | ARDRNMRGLINKK <b>FYYYYGMDV<sup>b</sup></b><br>(WGQGTTLTVSS)   | 4                              | 0     | ELISA                                 |
|         | IGHV1-69*09                    | ASNNLTGTTVTYSYGMVDV<br>(WGQGTTLTVSS)                          | 2                              | 0     | ELISA                                 |
|         | <b>IGHV3-48*04<sup>a</sup></b> | ARGRTTFGGEGQLFDY<br>(WGQGTTLTVSS)                             | 9                              | 9     | Alternate panning, ELISA              |
|         | IGHV4-34*01                    | ARGLDFRSGYSSSDGDYDSYYVDV<br>(WGKGTTTVSS)                      | 7                              | 8     | Alternate panning, ELISA              |
|         | IGHV4-59*08                    | AGGYCNSTSCYNQAFYS <b>YYYYVDL<sup>b</sup></b><br>(WGTTGTTTVSS) | 2                              | 4     | ELISA                                 |
|         | IGHV7-4-1*02                   | AKGGDYGDFD(WGPGTLTVSS)                                        | 7                              | 6     | Alternate panning, ELISA              |
|         | Total                          |                                                               | 31                             | 27    |                                       |
|         | JLDO                           |                                                               |                                |       |                                       |
| JLDO    | IGHV1-18*01                    | ARDLPTVVTPAPFDY(WGQGTTLTVSS)                                  | 3                              | 3     | Alternate panning, ELISA              |
|         | IGHV1-69*09                    | ASPYSGYDFLWAY(WGQGTTLTVSS)                                    | 5                              | 2     | ELISA                                 |
|         | IGHV3-23*01                    | AKWDHYPTAFDI(WGQGTMTVPS)                                      | 12                             | 6     | Alternate panning, ELISA              |
|         | IGHV4-34*01                    | ASRPLYCSGGNCYSYYQYGMNV<br>(WGQGTTLTVSS)                       | 5                              | 3     | Alternate panning, ELISA              |
| Total   |                                |                                                               | 25                             | 14    |                                       |

<sup>a</sup>Those mAbs in the VH column presented in bold were also identified previously using hybridoma method (40).

<sup>b</sup>The D/ExxxW amino acid motif and YYY aromatic amino acid clusters in CDR3 of four independent clones are in boldface.

<sup>c</sup>Those mAbs in italics were only identified from either Dsg1 or LJM11 panning.

<sup>d</sup>The cross-reactivity of these mAbs was confirmed by anti-Dsg1 and anti-LJM11 ELISA.

<sup>e</sup>The cross-reactivity of these mAbs was confirmed by Dsg1/LJM11 alternate panning.

ELISA (40, 44). All ELISA tested mAbs are cross-reactive (Table I and data not shown). Three independent clones isolated from either Dsg1 or LJM11 individual panning (one from GCDS and two from FGS; in italics in Table I) also cross-react to both Dsg1 and LJM11. Thus, all of the clones identified from our panning were cross-reactive based on one or the combination of two methods, and the results are summarized in Table I. Accordingly, despite differences in their mutations in CDRs and FWRs of those mAbs in each individual clonal lineage (see below and Fig. 1), their specificity to both Dsg1 and LJM11 is conserved.

#### Clonal selection and expansion among anti-Dsg1/LJM11 mAbs

Studies on some autoimmune diseases suggest that the autoimmune response in these diseases involves clonal selection and expansion events and is often oligoclonal (52, 53). In the present investigation, we isolated a total of 14 independent clones from 136 Dsg/LJM11-specific clones. It appears that autoimmune response within each individual patient is oligoclonal because only a limited number of independent clones were isolated. Some of the mAbs used the same VH genes by different FS patients. For example, IGHV1-18, IGHV1-69, and IGHV4-34 are shared by patients FGS and JLDO, with different CDR3 sequences between each lineage of mAbs from these two patients (Table I). Some of the mAbs from two patients share the same VH genes, suggesting there is a level of constrained VH gene usage in FS, consistent with other studies on the Ab repertoire in autoimmune diseases (53). The analyses of VH genes of these mAbs identified from panning of IgG4 APD libraries also demonstrate that the clonal expansion is apparent in all three patients. Phylogenetic trees in Fig. 1 suggest that evolutionarily these clones originated from the same parental B cells. For example, in the *top left panel*, all of

these Abs from patient GCDS are all encoded by IGHV5-51 VH gene and have the same CDR3 sequence. The VH genes of these Abs originated from the germline gene IGHV5-51 and underwent somatic mutation and clonal expansion during their maturations (see below). It is apparent that there is no divergent development among these mAbs identified from panning against either Dsg1 (clones initiated with D in Fig. 1) or LJM11 (clones initiated with L in Fig. 1). Most of the Dsg1 and LJM11 clones can be found on the same branches, further suggesting that Dsg1 and LJM11 have shared epitope(s), and both may serve as the selecting Ag for driving the development of these Abs.

It is interesting that the H-CDR3 of two independent clones from one patient (GCDS) have D/ExxxW consensus sequences (in boldface in Table I), which is the same as shared amino acid sequences in pathogenic pemphigus mAbs (50). Two H-CDR3 of independent clones from another patient also have YYYYY aromatic amino acid clusters (in boldface in Table I), similar to a pathogenic mAb from another study (46).

#### Extensive mutations among anti-Dsg1/LJM11 IgG4 Abs in FS patients

As it has been shown, those mutations are not the results of PCR errors through amplification of H- and L-chain V genes during APD library constructions (47). To further determine whether there are any characteristic mutations of anti-Dsg1 and anti-LJM11 IgG4 Abs in FS during their evolution, the somatic mutations of five lineages of mAb clones were identified using IgBlast and aligned (Fig. 2). All mAbs isolated from the libraries are extensively mutated, both in their CDRs and FWRs (Fig. 2). Similar to those phylogenetic analyses shown in Fig. 1, the different mutations among different clones in the same lineages of mAbs (using the same VH genes) demonstrate clear stepwise mutation during their

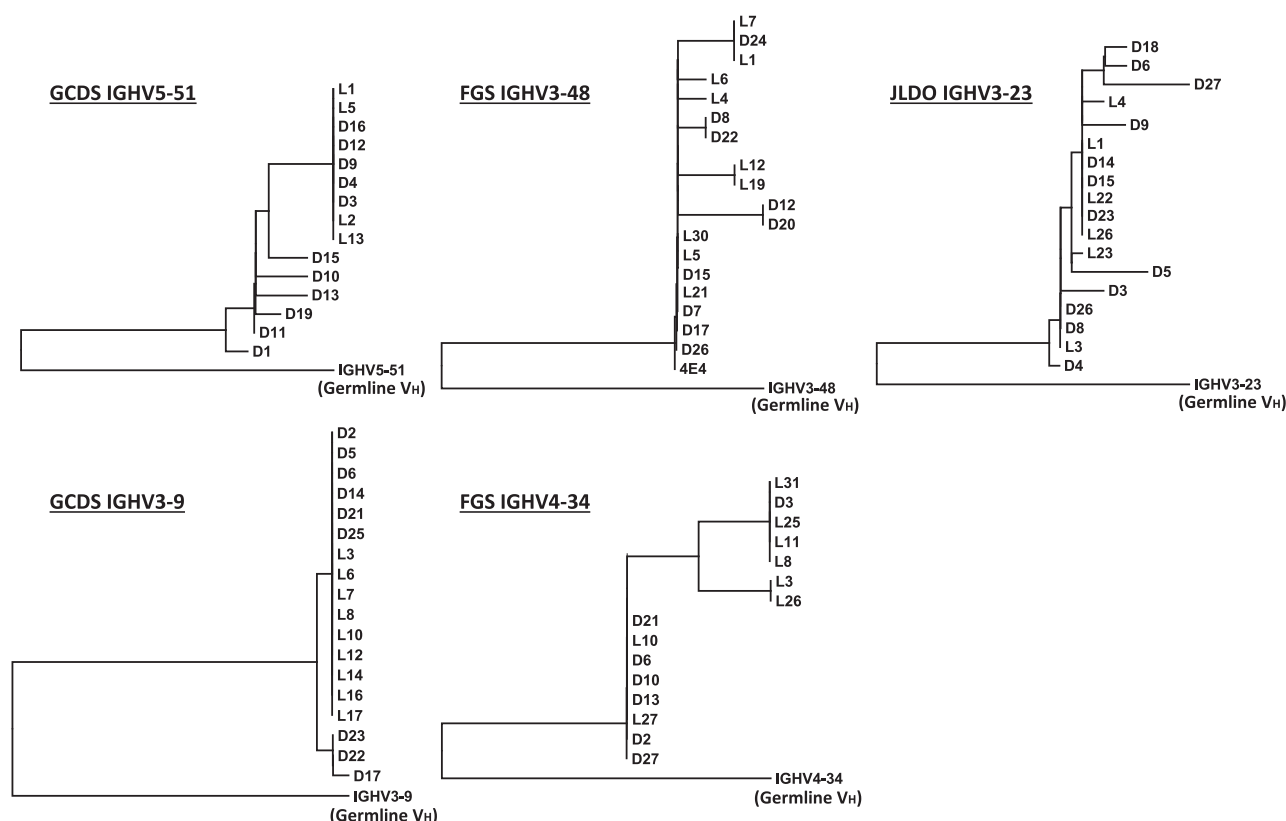


**FIGURE 1.** Phylogenetic tree of clonally related IgG4 Abs isolated from panning of IgG4 ADP libraries. Clone names starting with D indicate these clones were identified from Dsg1 panning, whereas those starting with L indicate they were identified from LJM11 panning.

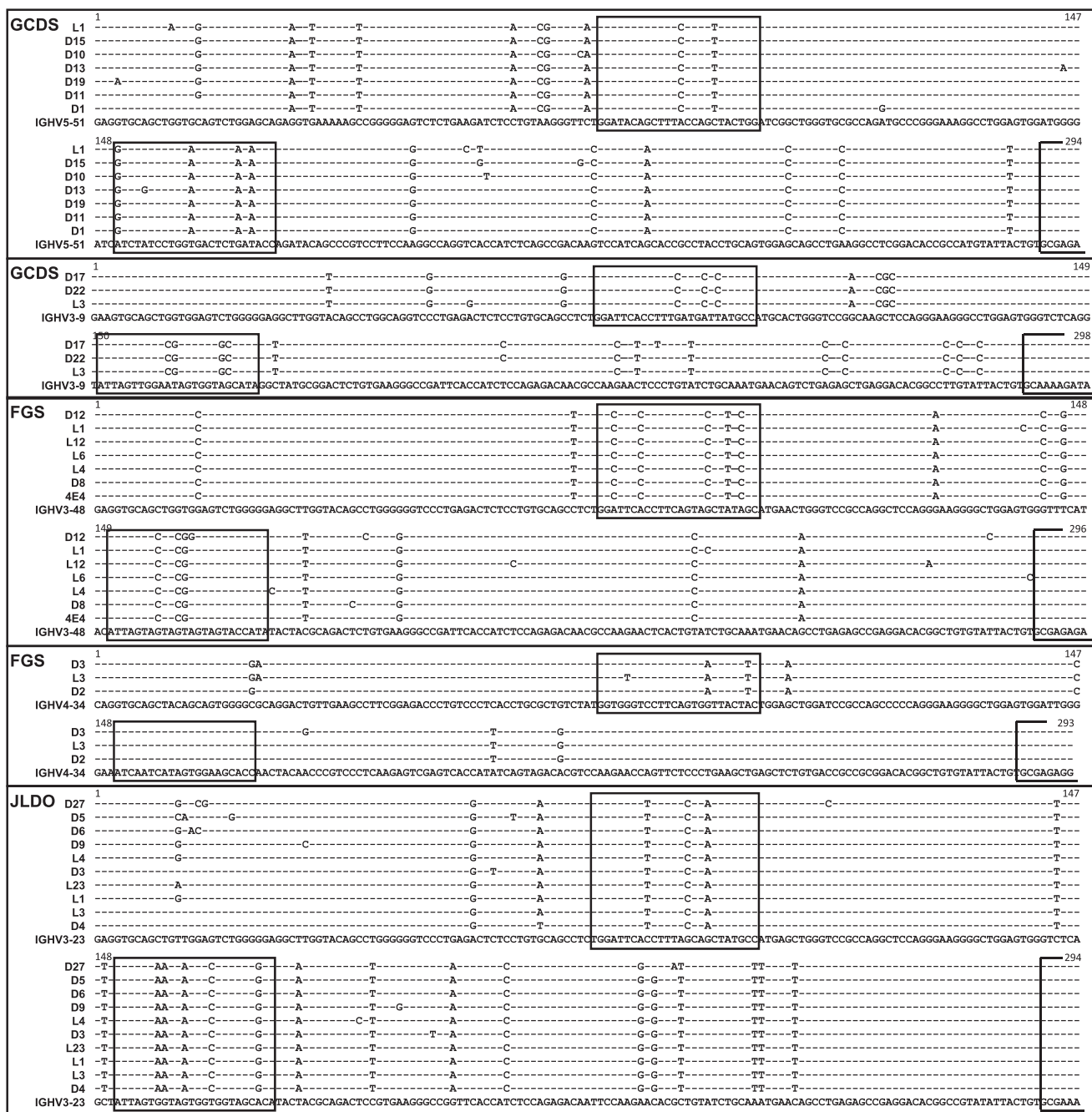


**FIGURE 2.** Alignments of clonally related IgG4 Abs identified from IgG4 ADP libraries. CDR1, CDR2, and partial CDR3 sequences are boxed. Mutated nucleotides are noted above the germline sequences. One representative clone is selected for those clones that have the identical VH gene sequences.

evolutions, consistent with continuous antigenic stimulation. One striking consistency is that despite extensive different FWR mutations among those different clones of the same clonal lineage, almost all of the mutations at CDR regions are preserved. For example, clones from IGHV3-23 lineage of JLDO shown in Fig. 2 (*bottom panel*), all mutations at CDR1 and CDR2 (boxed) are identical (C63T, G89C, and G92A in CDR1 and G15157A, G158A, G161A, T165C, and A172G in CDR2). In contrast, there is at least one mutation difference at FWRs for these listed clones using the same VH gene (IGHV3-23). In addition, as shown in Fig. 3 using GCDS-L3 as an example, except one mutation at CDR2 (T-C mutation), all other mutations at CDR1 and CDR2 are replacement mutations, which result in the change of amino acids compared with the germline VH (IGHV3-9). Those highly preserved replacement mutations at

CDRs suggest that they were specifically favored during affinity maturation of these mAbs during their evolution. The average ratios of R/S in CDRs are 2.47, 2.27, and 7.92, respectively, for the three FS patients, which is significantly higher than that in FWR (1.08, 1.17, and 1.26) ( $\chi^2$  test,  $p < 0.001$ ) (Table II). The CDR R/S ratios of 10 clones (in boldface in Table II), out of a total of 14 independent clones, are  $>2.9$ , which also indicates Ag selection (54). Highly diverse nucleotide mutations on the same VH clone clusters suggests that these mAbs underwent multiple rounds of clonal expansion, consistent with our hypothesis that chronic antigenic stimulation (by either self-antigen or environmental Ag, or both) is a feature for FS Ab development.

Shared mutations have been found previously in CDRs of the same VH genes encoding anti-Dsg1 mAbs among FS patients and

|                |                                                                                             |                        |               |
|----------------|---------------------------------------------------------------------------------------------|------------------------|---------------|
| <b>GCDS-L3</b> | E S G G G L V Q P G R S L R L S C A G S                                                     | <b>G F T F H A H A</b> | M H           |
|                | GAGTCTGGGGGAGGCTTGGTTTCAGCCTGGCAGGTGCGTGAGGCTCTCCTGTGCAGGCTCTGGATTACCTTTTCATGCTCATGCCATGCAC |                        |               |
| <b>IGHV3-9</b> | .....A.....C.....A.....C.....G...A.T.....                                                   |                        |               |
|                | E S G G G L V Q P G R S L R L S C A <u>A</u> S                                              | <b>G F T F D D Y A</b> | M H           |
| <b>GCDS-L3</b> | W V R Q R P G K G L E W V S G                                                               | <b>I S W T S G A I</b> | G Y A D S V K |
|                | TGGGTCCGACACGCCAGGGAAGGGCCTGGAGTGGGTCTCAGGTATATAGTTGGACGAGTGGTGCCATAGTTATGCGGACTCTGTGAAG    |                        |               |
| <b>IGHV3-9</b> | .....G...GCT.....AT.....AG.....C.....                                                       |                        |               |
|                | W V R Q <u>A</u> P G K G L E W V S G                                                        | <b>I S W N S G S I</b> | G Y A D S V K |
| <b>GCDS-L3</b> | G R F T I S R D N A N N S L F L Q M N S L R P E D T A L Y Y                                 |                        |               |
|                | GGCCGATTACCATCTCCAGAGACAACGCCAACAATTCCTGTTTCTGCAAATGAACAGTCTGCGACCTGAGGACACGGCCCTCTACTAC    |                        |               |
| <b>IGHV3-9</b> | .....G..C.....A.....A..G.....T.G..T...                                                      |                        |               |
|                | G R F T I S R D N A <u>K</u> N S L <u>Y</u> L Q M N S L R <u>A</u> E D T A L Y Y            |                        |               |

**FIGURE 3.** Cross-reactive IgG4 mAb genes are extensively mutated, especially in CDRs (boxed) compared with FWRs. The H-chain gene of a representative clone is aligned with its germline gene. Amino acids from germline sequence changed as a result of replacement mutations are underlined.

an individual before the onset of FS (40). To determine whether particular shared mutations are necessary for the development of the Dsg1/LJM11 specificity, the three shared VH genes (IGHV1-18, IGHV1-69, and IGHV4-34) from two FS patients (FGS and JLDO) were aligned to identify shared mutation among them. However, no shared mutation is present among all these VH gene-sharing mAbs between those from two patients (Fig. 4). Furthermore, the CDR3 sequences of these VH gene-sharing mAbs from two patients are also different, as shown in Table I. In general, H-chain CDR3 is important for Ab specificity (55, 56). Though our result suggests that the Dsg1/LJM11 specificity of cross-reactive Abs from FS may be governed by the combination of all three CDR sequences rather than by a single CDR such as CDR3. This suggests that the Ab response in different individuals is flexible and may have multiple routes to achieve the same specificity during the positive selection of their respective Ab evolution. The extensive mutations in FWRs among those FS autoantibodies (Table II) suggest that those mutations may also aid in improving the affinity of these autoantibodies to their target Ags in FS, as found previously in broadly neutralizing Abs to HIV-1 (57).

#### Germline VH and VL encoded revertant mAbs from FS recognize both Dsg1 and LJM11

The wide presence of Dsg1 and LJM11 cross-reactive Abs in FS suggests that either Dsg1 or LJM11 or both may participate in driving the development of these Abs. However, it does not reveal

whether Dsg1 or LJM11 could be the initial target for the naive B cells in FS. It is also possible that some other Ags (either environmental Ag or self-antigen), but not Dsg1 or LJM11, are the initial target that activates the naive B cells, which are the precursors for the eventual plasma cells that secrete these autoreactive Abs in FS. Therefore, it would be interesting to determine if naive B cells expressing germline V genes can bind Dsg1 and LJM11.

The mutations in the VH and VL genes of two cross-reactive mAbs, namely FGS-4E4 and GCDS-2D11 (their VH are in boldface in Table I), which were found using both the hybridoma method (13, 40) and APD method, were identified using IgBlast (45) and reverted back to the germline configurations (Fig. 5A). The DNA of the germline VH and VL genes of these two revertant mAbs were synthesized, subcloned into the original modified pComb3xSS vector (see *Materials and Methods*), produced, and purified as scFv (4E4-R and 2D11-R). The reactivity of these two revertant mAbs to Dsg1 and LJM11 was tested by ELISA and compared with that of their mutated counterpart scFv. As shown in Fig. 5B, these two revertant scFv react to both Dsg1 and LJM11. This suggests that either Dsg1 or LJM11 (or both) may be the initial target(s) of autoimmune responses in FS, further implicating the environmental contribution to the development of FS.

## Discussion

Our previous studies regarding the presence of cross-reactive mAbs in FS patients (13) suggest that there might be an overlap between

**Table II.** R/S in CDRs and FWRs of VH genes of the IgG4 scFv from FS patients

| Patient | VH                 | Total Clones | CDR            | FWR (R/S)      |
|---------|--------------------|--------------|----------------|----------------|
| GCDS    | <b>IGHV2-5*021</b> | <b>4</b>     | <b>12/4</b>    | <b>8/10</b>    |
|         | <b>IGHV3-9*01</b>  | <b>18</b>    | <b>108/18</b>  | <b>112/183</b> |
|         | IGHV3-53*04        | 2            | 12/6           | 36/16          |
|         | IGHV5-51*03        | 15           | 46/44          | 144/68         |
|         | <b>Total</b>       | <b>39</b>    | <b>178/72</b>  | <b>300/277</b> |
| FGS     | <b>IGHV1-18*01</b> | <b>4</b>     | <b>24/7</b>    | <b>42/24</b>   |
|         | <b>IGHV1-69*09</b> | <b>2</b>     | <b>6/0</b>     | <b>17/7</b>    |
|         | IGHV3-48*04        | 18           | 119/76         | 108/36         |
|         | <b>IGHV4-34*01</b> | <b>15</b>    | <b>33/0</b>    | <b>34/45</b>   |
|         | <b>IGHV4-59*08</b> | <b>6</b>     | <b>24/1</b>    | <b>59/34</b>   |
|         | IGHV7-4-1*02       | 13           | 39/24          | 49/119         |
|         | <b>Total</b>       | <b>58</b>    | <b>245/108</b> | <b>309/265</b> |
| JLDO    | <b>IGHV1-18*01</b> | <b>6</b>     | <b>12/0</b>    | <b>18/20</b>   |
|         | <b>IGHV1-69*09</b> | <b>7</b>     | <b>28/6</b>    | <b>34/5</b>    |
|         | <b>IGHV3-23*01</b> | <b>18</b>    | <b>126/18</b>  | <b>141/130</b> |
|         | <b>IGHV4-34*01</b> | <b>8</b>     | <b>32/1</b>    | <b>17/12</b>   |
|         | <b>Total</b>       | <b>39</b>    | <b>198/25</b>  | <b>210/167</b> |

The CDR R/S ratios of 10 clones are presented in bold.

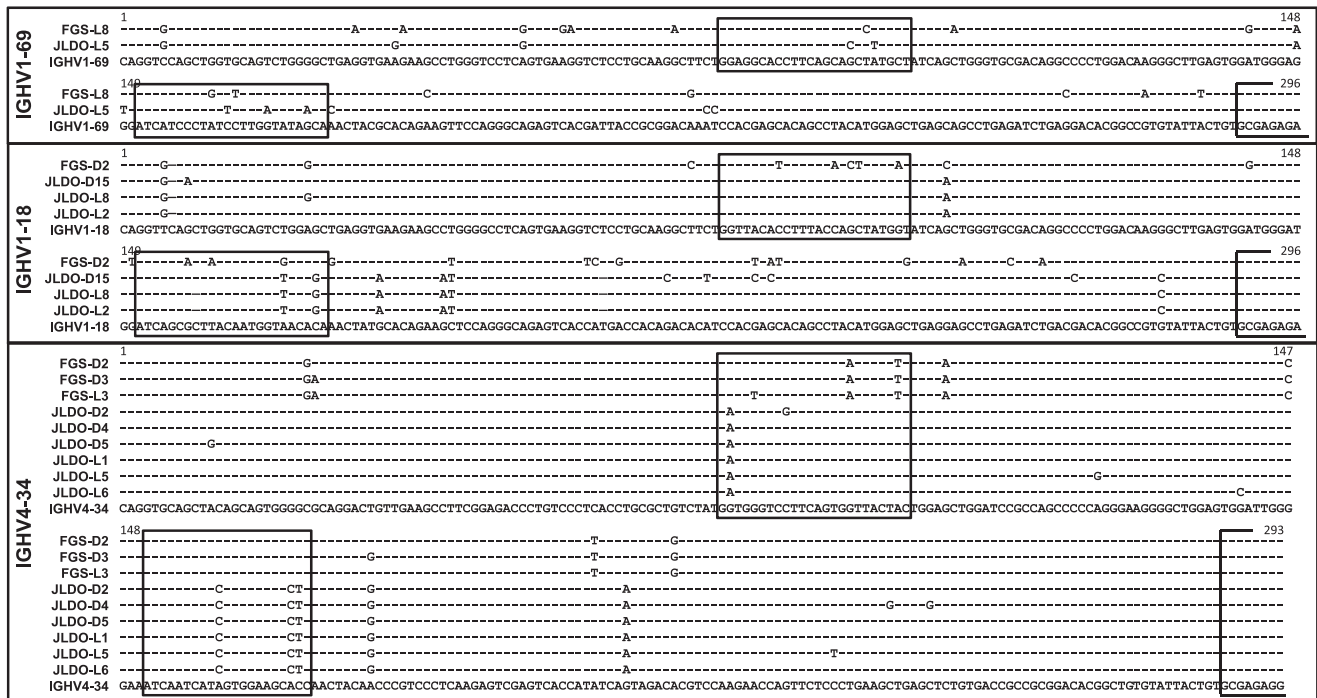


**FIGURE 4.** Alignments of IgG4 Ab clusters that use the same VH genes identified from two different patients. CDR1, CDR2, and partial CDR3 sequences are boxed. All nucleotide mutations are noted above the germline sequences.

the immune responses toward these two Ags. In this investigation, we seek to disclose the presence of an overlap between IgG4 responses against either Dsg1 or LJM11 in FS. Based on our hypothesis, the cross-reactive epitope(s) on Dsg1 and LJM11 would drive the development of cross-reactive Abs in FS. Due to the polyclonal nature of the Abs in patients' sera, the cross-reactivity of those IgG4 Abs can only be determined using monoclonal IgG4 Abs. The construction of IgG4-specific APD libraries and the subsequent panning of these libraries against either autoantigen or environmental Ag (i.e., LJM11 sand fly Ag) make it possible to identify these Ag-specific IgG4 Abs. Compared to the hybridoma method used in our previous investigation on FS Ab development (40), our newly developed IgG4 APD method modified from original APD method (41) has a distinct advantage over the hybridoma method. For example, only two IgG4 mAbs were isolated using hybridoma method and even several thousands of hybridoma clones were screened from eight FS patient B cell samples (40) (Y. Qian, unpublished observations). However, 14 IgG4 independent mAb clones are isolated from 3 FS patients using this IgG4 APD method. It indicates that IgG4 APD method has broader coverage of the Ab repertoire than hybridoma method. Moreover, this newly developed IgG4 APD method may have a wider application to the study of IgG4 Ab repertoires of other IgG4-related diseases in the future.

The presence of cross-reactive mAbs in FS (13) also suggests the presence of cross-reactive epitope(s) between autoantigen Dsg1 and environmental Ag LJM11. Notably, these two molecules are evolutionary distant and share little primary sequence homology. The cross-reactive mAbs from FS patients bind both molecules via conformational epitope (13), which does not rely on the similarity of their linear sequences. This explains the possible presence of a cross-reactive epitope on both molecules and also suggests that there are other epitopes on both molecules that are not cross-reactive, such as those epitopes that consist of continuous amino acids (linear epitopes). Thus, besides these Abs derived against the

cross-reactive epitope(s) on both molecules, it should be expected that there are Abs from FS patients that are either Dsg1- or LJM11-specific that do not cross-react to the other. It is quite surprising that all mAbs identified from our investigation are cross-reactive, regardless of whether they are obtained from panning against Dsg1 or LJM11. Even though it might be unlikely, we cannot rule out that these two molecules share more than one conformational epitope. Hence, it is possible that these mAbs bind different cross-reactive epitopes shared by these two molecules. Still, there might be other explanations. For example, the current screening method for mAb clones identified by panning of APD libraries only allows us to examine a limited number of clones. Thus, it is possible that only the most abundant clones were captured, whereas those rare clones, such as Dsg1- or LJM11-specific, escaped the detection. Nevertheless, the detection of identical and clonally related clones indicates that the most abundant IgG4 mAbs in these FS patients have been disclosed, and the presence of these cross-reactive IgG4 demonstrates the possible contribution of the environmental Ag in FS development. The rapid development of next generation sequencing method for high-throughput Ab sequence analysis will make it possible to identify those rare clones if the need arises in the future (58).

Another implication of our finding is that the cross-reactive epitope(s) might be the most immunogenic (or most dominant) that induces the strongest immune response against this epitope(s), as the conservation of the CDR mutations among most VH sharing mAbs identified from the same patient is remarkably stable. Thus, most IgG4 Abs from FS patients might be developed against this epitope. LJM11 is known to be the most immunogenic component in sand fly saliva (35), and it might be due to this cross-reactive epitope. In this regard, the cross-reactive epitope on Dsg1 must be a predominant epitope for the development of anti-Dsg1 Abs in FS. The presence of a predominant epitope is corroborated by other investigations on pemphigus. For instance, it has been shown that one predominant pathogenic epitope on Dsg1 may be targeted

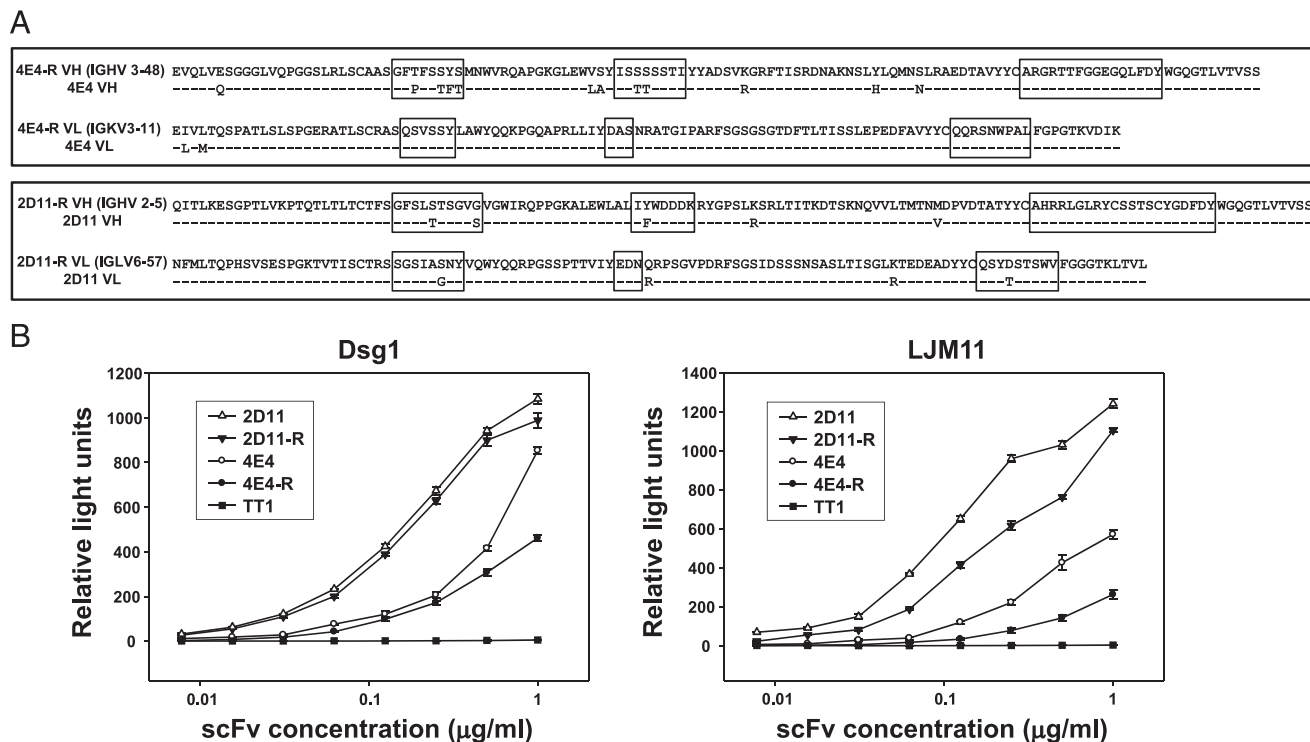


**FIGURE 5.** Revertant mAbs derived from two cross-reactive anti-Dsg1 mAbs from FS also recognize both Dsg1 and LJM11. **(A)** H- and L-chain amino acid sequences of 4E4 (*top panel*) and 2D11 (*bottom panel*) and their corresponding revertant counterparts (encoded by germline VH and VL genes). Only mutated amino acids are shown for the VH and VL chains of 4E4 and 2D11. The amino acid sequences of CDR1, -2, and -3 of VH and VL are boxed. **(B)** Similar to mutated anti-Dsg1 IgG4 scFv mAbs, their revertant scFv mAbs also react to both Dsg1 (*left panel*) and LJM11 (*right panel*). The negative control scFv (TT1) does not react with either Dsg1 or LJM11. The reactivity of each scFv Abs to either Dsg1 (*left panel*) or LJM11 (*right panel*) is measured by relative light units (RLU) as previously described (36). The results shown are representative of three independent experiments with triplicates in each experimental group.

by most PF patients, as the binding of an anti-Dsg1 pathogenic mAb can be inhibited by sera from these patients (48). Similarly, there is also an immunodominant epitope on Dsg3 in mucocutaneous pemphigus vulgaris (PV) (46). Not only is the binding of a pathogenic mAb from a PV patient to Dsg3 inhibited by these tested PV sera, but this pathogenic mAb also inhibits PV polyclonal Abs from binding to Dsg3 (46). Therefore, it is possible that all of these mAbs isolated from this study target one conformational epitope shared by Dsg1 and LJM11 or epitopes in the vicinity of or overlapping this epitope.

The isolated cross-reactive mAbs could be either pathogenic or not pathogenic. If these IgG4 autoantibodies are pathogenic, it would indicate that an environmental Ag may be directly involved in driving the pathogenic IgG4 autoantibody response in FS. If they are not pathogenic, it would suggest that these IgG4 autoantibodies may play a role in activating an extended autoantibody response, via mechanisms such as epitope spreading in FS, and this subsequent autoantibody response leads to the development of pathogenic autoantibodies in FS, as demonstrated before (59). In either case, the inciting Ag(s) (Dsg1 and/or LJM11) would be directly and/or indirectly involved in the development of pathogenic IgG4 autoantibodies in FS.

The current study also demonstrates that a noninfectious environmental Ag may contribute to the initiation of an autoimmune disease. It is possible, as shown in a previous study (36), that these FS genetically susceptible individuals mount an IgE response to sand fly Ag exposure. They then develop IgG4 Abs in response to the repeated (chronic) exposure to sand fly bites, because of the well-known transition from IgE to IgG4 during chronic antigenic stimulation or allergen-specific immunotherapy in allergy (60–

63). These IgG4 Abs turn out to be cross-reactive to Dsg1 autoantigen. Those anti-Dsg1 IgG4 Abs themselves or their triggered subsequent development of additional anti-Dsg1 IgG4 Abs result in the development of FS in those genetically susceptible individuals.

It is noteworthy that reverting mutated IgG4 autoantibodies from FS back to their germline VH and VL genes do not abolish their reactivity to both Dsg1 and LJM11. Numerous studies using revertant Abs to study human autoimmune diseases, such as pemphigus vulgaris and systemic lupus erythematosus indicate that the revertant Abs lose their reactivity (46, 64) or have reduced reactivity (65) to their corresponding autoantigens once their H- and L-chain V regions reverted back to their germline formats. Autoantibody development from non-autoreactive B cells has been proposed (64, 65). Due to the lack of candidate antigenic stimulants for triggering the development of these autoantibodies in human autoimmune diseases, the initial targets (except these autoantigens of their corresponding autoimmune diseases) for these naive B cells could not be further identified using revertant mAb method. In this study, we provide evidence that environmental Ags, such as LJM11 from sand fly, could be an antigenic stimulant that activates the naive B cells in those individuals who are genetically predisposed to FS disease and the subsequent development of IgG4 autoreactive Abs.

In summary, the extensive overlap of the IgG4 responses toward both Dsg1 autoantigen and LJM11 environmental Ag in this study is consistent with our hypothesis and thus establishes the possible link between the exposure to a noninfectious environmental Ag and the development of IgG4 autoantibodies in FS. Moreover, that revertant mAbs also cross-react to both

autoantigen (Dsg1) and environmental Ag (LJM11) provides further evidence that LJM11 could be the environmental Ag that triggers the activation of naive B cells. All of this evidence strongly suggests that LJM11 component from sand fly saliva as an environmental Ag may trigger the development of IgG4 autoantibodies in FS susceptible individuals in FS endemic regions. Our findings regarding the role of IgG4 response to an exogenous Ag in FS may be expanded to the understanding of the etiology of other IgG4-related autoimmune diseases.

## Disclosures

The authors have no financial conflicts of interest.

## References

- Diaz, L. A., S. A. Sampaio, E. A. Rivitti, C. R. Martins, P. R. Cunha, C. Lombardi, F. A. Almeida, R. M. Castro, M. L. Macca, C. Lavrado, et al. 1989. Endemic pemphigus foliaceus (Fogo Selvagem): II. Current and historic epidemiologic studies. *J. Invest. Dermatol.* 92: 4–12.
- Empinotti, J. C., L. A. Diaz, C. R. Martins, E. A. Rivitti, S. A. Sampaio, C. Lombardi, and J. A. Sanches, Cooperative Group for Fogo Selvagem Research. 1990. Endemic pemphigus foliaceus in western Parana, Brazil (1976–1988). *Br. J. Dermatol.* 123: 431–437.
- Lever, W. F. 1953. Pemphigus. *Medicine (Baltimore)* 32: 1–123.
- Beutner, E. H., and R. E. Jordon. 1964. Demonstration of Skin Antibodies in Sera of Pemphigus Vulgaris Patients by Indirect Immunofluorescent Staining. *Proc. Soc. Exp. Biol. Med.* 117: 505–510.
- Udey, M. C., and J. R. Stanley. 1999. Pemphigus—diseases of antidesmosomal autoimmunity. *JAMA* 282: 572–576.
- Anhalt, G. J., and L. A. Diaz. 2001. Prospects for autoimmune disease: Research advances in pemphigus. *JAMA* 285: 652–654.
- Amagai, M. 2003. Desmoglein as a target in autoimmunity and infection. *J. Am. Acad. Dermatol.* 48: 244–252.
- Ding, X., V. Aoki, J. M. Mascaro, Jr., A. Lopez-Swiderski, L. A. Diaz, and J. A. Fairley. 1997. Mucosal and mucocutaneous (generalized) pemphigus vulgaris show distinct autoantibody profiles. *J. Invest. Dermatol.* 109: 592–596.
- Diaz, L. A., S. A. Sampaio, E. A. Rivitti, C. R. Martins, P. R. Cunha, C. Lombardi, F. A. Almeida, R. M. Castro, M. L. Macca, C. Lavrado, et al. 1989. Endemic pemphigus foliaceus (fogo selvagem). I. Clinical features and immunopathology. *J. Am. Acad. Dermatol.* 20: 657–669.
- Stanley, J. R., V. Klaus-Kovtun, and S. A. Sampaio. 1986. Antigenic specificity of fogo selvagem autoantibodies is similar to North American pemphigus foliaceus and distinct from pemphigus vulgaris autoantibodies. *J. Invest. Dermatol.* 87: 197–201.
- Moraes, M. E., M. Fernandez-Vina, A. Lazaro, L. A. Diaz, G. H. Filho, H. Friedman, E. Rivitti, V. Aoki, P. Stastny, and J. R. Moraes. 1997. An epitope in the third hypervariable region of the DRB1 gene is involved in the susceptibility to endemic pemphigus foliaceus (fogo selvagem) in three different Brazilian populations. *Tissue Antigens* 49: 35–40.
- Aoki, V., R. C. Millikan, E. A. Rivitti, G. Hans-Filho, D. P. Eaton, S. J. Warren, N. Li, J. Hilario-Vargas, R. G. Hoffmann, and L. A. Diaz, Cooperative Group for Fogo Selvagem Research. 2004. Environmental risk factors in endemic pemphigus foliaceus (fogo selvagem). *J. Invest. Dermatol. Symp. Proc.* 9: 34–40.
- Qian, Y., J. S. Jeong, M. Maldonado, J. G. Valenzuela, R. Gomes, C. Teixeira, F. Evangelista, B. Qaqish, V. Aoki, G. Hans, Jr., et al. 2012. Cutting Edge: Brazilian pemphigus foliaceus anti-desmoglein 1 autoantibodies cross-react with sand fly salivary LJM11 antigen. *J. Immunol.* 189: 1535–1539.
- Goodnow, C. C. 2007. Multistep pathogenesis of autoimmune disease. *Cell* 130: 25–35.
- Fierabracci, A. 2009. Unravelling the role of infectious agents in the pathogenesis of human autoimmunity: the hypothesis of the retroviral involvement revisited. *Curr. Mol. Med.* 9: 1024–1033.
- Chervonsky, A. V. 2010. Influence of microbial environment on autoimmunity. *Nat. Immunol.* 11: 28–35.
- Miller, F. W. 2011. Environmental agents and autoimmune diseases. *Adv. Exp. Med. Biol.* 711: 61–81.
- Miller, F. W., L. Alfredsson, K. H. Costenbader, D. L. Kamen, L. M. Nelson, J. M. Norris, and A. J. De Roos. 2012. Epidemiology of environmental exposures and human autoimmune diseases: findings from a National Institute of Environmental Health Sciences Expert Panel Workshop. *J. Autoimmun.* 39: 259–271.
- Germolec, D., D. H. Kono, J. C. Pfau, and K. M. Pollard. 2012. Animal models used to examine the role of the environment in the development of autoimmune disease: findings from an NIEHS Expert Panel Workshop. *J. Autoimmun.* 39: 285–293.
- Rock, B., C. R. Martins, A. N. Theofilopoulos, R. S. Balderas, G. J. Anhalt, R. S. Labib, S. Futamura, E. A. Rivitti, and L. A. Diaz. 1989. The pathogenic effect of IgG4 autoantibodies in endemic pemphigus foliaceus (fogo selvagem). *N. Engl. J. Med.* 320: 1463–1469.
- Warren, S. J., L. A. Arteaga, E. A. Rivitti, V. Aoki, G. Hans-Filho, B. F. Qaqish, M. S. Lin, G. J. Giudice, and L. A. Diaz. 2003. The role of subclass switching in the pathogenesis of endemic pemphigus foliaceus. *J. Invest. Dermatol.* 120: 104–108.
- Qaqish, B. F., P. Prisanh, Y. Qian, E. Andraca, N. Li, V. Aoki, G. Hans-Filho, V. dos Santos, E. A. Rivitti, and L. A. Diaz, Cooperative Group on Fogo Selvagem Research. 2009. Development of an IgG4-based predictor of endemic pemphigus foliaceus (fogo selvagem). *J. Invest. Dermatol.* 129: 110–118.
- Sitaru, C., S. Mihai, and D. Zillikens. 2007. The relevance of the IgG subclass of autoantibodies for blister induction in autoimmune bullous skin diseases. *Arch. Dermatol. Res.* 299: 1–8.
- Kamisawa, T., and A. Okamoto. 2006. Autoimmune pancreatitis: proposal of IgG4-related sclerosing disease. *J. Gastroenterol.* 41: 613–625.
- Yamamoto, M., H. Takahashi, S. Sugai, and K. Imai. 2005. Clinical and pathological characteristics of Mikulicz's disease (IgG4-related plasmacytic exocrinopathy). *Autoimmun. Rev.* 4: 195–200.
- Stone, J. H., Y. Zen, and V. Deshpande. 2012. IgG4-related disease. *N. Engl. J. Med.* 366: 539–551.
- Stone, J. H., A. Khosroshahi, V. Deshpande, J. K. Chan, J. G. Heathcote, R. Aalberse, A. Azumi, D. B. Bloch, W. R. Brugge, M. N. Carruthers, et al. 2012. Recommendations for the nomenclature of IgG4-related disease and its individual organ system manifestations. *Arthritis Rheum.* 64: 3061–3067.
- Tokura, Y., H. Yagi, H. Yanaguchi, Y. Majima, A. Kasuya, T. Ito, M. Maekawa, and H. Hashizume. 2014. IgG4-related skin disease. *Br. J. Dermatol.* 171: 959–967.
- Roscoe, J. T., L. Diaz, S. A. Sampaio, R. M. Castro, R. S. Labib, Y. Takahashi, H. Patel, and G. J. Anhalt. 1985. Brazilian pemphigus foliaceus autoantibodies are pathogenic to BALB/c mice by passive transfer. *J. Invest. Dermatol.* 85: 538–541.
- Anhalt, G. J., R. S. Labib, J. J. Voorhees, T. F. Beals, and L. A. Diaz. 1982. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. *N. Engl. J. Med.* 306: 1189–1196.
- Gourley, M., and F. W. Miller. 2007. Mechanisms of disease: Environmental factors in the pathogenesis of rheumatic disease. *Nat. Clin. Pract. Rheumatol.* 3: 172–180.
- Cooper, G. S., K. M. Gilbert, E. L. Greidinger, J. A. James, J. C. Pfau, L. Reinlib, B. C. Richardson, and N. R. Rose. 2008. Recent advances and opportunities in research on lupus: environmental influences and mechanisms of disease. *Environ. Health Perspect.* 116: 695–702.
- Münz, C., J. D. Lünemann, M. T. Getts, and S. D. Miller. 2009. Antiviral immune responses: triggers of or triggered by autoimmunity? *Nat. Rev. Immunol.* 9: 246–258.
- Francis, L., and A. Perl. 2010. Infection in systemic lupus erythematosus: friend or foe? *Int. J. Clin. Rheumatol.* 5: 59–74.
- Teixeira, C., R. Gomes, N. Collin, D. Reynoso, R. Jochim, F. Oliveira, A. Seitz, D. E. Elnaïem, A. Caldas, A. P. de Souza, et al. 2010. Discovery of markers of exposure specific to bites of *Lutzomyia longipalpis*, the vector of *Leishmania infantum* chagasi in Latin America. *PLoS Negl. Trop. Dis.* 4: e638.
- Qian, Y., J. S. Jeong, M. Abdeladhim, J. G. Valenzuela, V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz, Cooperative Group on Fogo Selvagem Research. 2015. IgE anti-LJM11 sand fly salivary antigen may herald the onset of fogo selvagem in endemic Brazilian regions. *J. Invest. Dermatol.* 135: 913–915.
- McClain, M. T., L. D. Heinlen, G. J. Dennis, J. Roebuck, J. B. Harley, and J. A. James. 2005. Early events in lupus humoral autoimmunity suggest initiation through molecular mimicry. *Nat. Med.* 11: 85–89.
- Cusick, M. F., J. E. Libbey, and R. S. Fujinami. 2012. Molecular mimicry as a mechanism of autoimmune disease. *Clin. Rev. Allergy Immunol.* 42: 102–111.
- Joseph, C. G., E. Darrah, A. A. Shah, A. D. Skora, L. A. Casciola-Rosen, F. M. Wigley, F. Boin, A. Fava, C. Thoburn, I. Kinde, et al. 2014. Association of the autoimmune disease scleroderma with an immunologic response to cancer. *Science* 343: 152–157.
- Qian, Y., S. H. Clarke, V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz. 2009. Antigen selection of anti-DSG1 autoantibodies during and before the onset of endemic pemphigus foliaceus. *J. Invest. Dermatol.* 129: 2823–2834.
- Barbas, C. F. I., D. R. Burton, J. K. Scott, and G. J. Silverman. 2001. *Phage display: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Efremov, D. G., F. D. Batista, and O. R. Burrone. 1993. Molecular analysis of IgE H-chain transcripts expressed in vivo by peripheral blood lymphocytes from normal and atopic individuals. *J. Immunol.* 151: 2195–2207.
- Xu, X., F. Oliveira, B. W. Chang, N. Collin, R. Gomes, C. Teixeira, D. Reynoso, V. My Pham, D. E. Elnaïem, S. Kamhawi, et al. 2011. Structure and function of a “yellow” protein from saliva of the sand fly *Lutzomyia longipalpis* that confers protective immunity against *Leishmania major* infection. *J. Biol. Chem.* 286: 32383–32393.
- Qian, Y., P. Prisanh, E. Andraca, B. F. Qaqish, V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz, Cooperative Group on Fogo Selvagem Research. 2011. IgE, IgM, and IgG4 anti-desmoglein 1 autoantibody profile in endemic pemphigus foliaceus (fogo selvagem). *J. Invest. Dermatol.* 131: 985–987.
- Ye, J., N. Ma, T. L. Madden, and J. M. Ostell. 2013. IgBLAST: an immunoglobulin variable domain sequence analysis tool. *Nucleic Acids Res.* 41: W34–W40.
- Di Zenzo, G., G. Di Lullo, D. Corti, V. Calabresi, A. Sinistro, F. Vanzetta, B. Didona, G. Cianchini, M. Hertl, R. Emig, et al. 2012. Pemphigus autoantibodies generated through somatic mutations target the desmoglein-3 cis-interface. *J. Clin. Invest.* 122: 3781–3790.
- Payne, A. S., K. Ishii, S. Kacir, C. Lin, H. Li, Y. Hanakawa, K. Tsunoda, M. Amagai, J. R. Stanley, and D. L. Siegel. 2005. Genetic and functional characterization of human pemphigus vulgaris monoclonal autoantibodies isolated by phage display. *J. Clin. Invest.* 115: 888–899.
- Ishii, K., C. Lin, D. L. Siegel, and J. R. Stanley. 2008. Isolation of pathogenic monoclonal anti-desmoglein 1 human antibodies by phage display of pemphigus foliaceus autoantibodies. *J. Invest. Dermatol.* 128: 939–948.

49. Yamagami, J., S. Kacir, K. Ishii, A. S. Payne, D. L. Siegel, and J. R. Stanley. 2009. Antibodies to the desmoglein 1 precursor proprotein but not to the mature cell surface protein cloned from individuals without pemphigus. *J. Immunol.* 183: 5615–5621.
50. Yamagami, J., A. S. Payne, S. Kacir, K. Ishii, D. L. Siegel, and J. R. Stanley. 2010. Homologous regions of autoantibody heavy chain complementarity-determining region 3 (H-CDR3) in patients with pemphigus cause pathogenicity. *J. Clin. Invest.* 120: 4111–4117.
51. Snow, R. E., C. J. Chapman, S. T. Holgate, and F. K. Stevenson. 1998. Clonally related IgE and IgG4 transcripts in blood lymphocytes of patients with asthma reveal differing patterns of somatic mutation. *Eur. J. Immunol.* 28: 3354–3361.
52. Link, J. M., and H. W. Schroeder, Jr. 2002. Clues to the etiology of autoimmune diseases through analysis of immunoglobulin genes. *Arthritis Res.* 4: 80–83.
53. Foreman, A. L., J. Van de Water, M. L. Gougeon, and M. E. Gershwin. 2007. B cells in autoimmune diseases: insights from analyses of immunoglobulin variable (Ig V) gene usage. *Autoimmun. Rev.* 6: 387–401.
54. Shlomchik, M. J., A. Marshak-Rothstein, C. B. Wolfowicz, T. L. Rothstein, and M. G. Weigert. 1987. The role of clonal selection and somatic mutation in autoimmunity. *Nature* 328: 805–811.
55. Mahmoud, T. I., H. W. Schroeder, Jr., and J. F. Kearney. 2011. Limiting CDR-H3 diversity abrogates the antibody response to the bacterial polysaccharide  $\alpha$  1 $\rightarrow$ 3 dextran. *J. Immunol.* 187: 879–886.
56. Silva-Sanchez, A., C. R. Liu, A. M. Vale, M. Khass, P. Kapoor, A. Elgavish, I. I. Ivanov, G. C. Ippolito, R. L. Schelonka, T. R. Schoeb, et al. 2015. Violation of an evolutionarily conserved immunoglobulin diversity gene sequence preference promotes production of dsDNA-specific IgG antibodies. *PLoS One* 10: e0118171.
57. Klein, F., R. Diskin, J. F. Scheid, C. Gaebler, H. Mouquet, I. S. Georgiev, M. Pancera, T. Zhou, R. B. Incesu, B. Z. Fu, et al. 2013. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell* 153: 126–138.
58. Ravn, U., G. Didelot, S. Venet, K. T. Ng, F. Gueneau, F. Rousseau, S. Calloud, M. Kosco-Vilbois, and N. Fischer. 2013. Deep sequencing of phage display libraries to support antibody discovery. *Methods* 60: 99–110.
59. Li, N., V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz. 2003. The role of intramolecular epitope spreading in the pathogenesis of endemic pemphigus foliaceus (fogo selvagem). *J. Exp. Med.* 197: 1501–1510.
60. Aalberse, R. C., R. van der Gaag, and J. van Leeuwen. 1983. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J. Immunol.* 130: 722–726.
61. Gleich, G. J., E. M. Zimmermann, L. L. Henderson, and J. W. Yunginger. 1982. Effect of immunotherapy on immunoglobulin E and immunoglobulin G antibodies to ragweed antigens: a six-year prospective study. *J. Allergy Clin. Immunol.* 70: 261–271.
62. Rossi, R. E., G. Monasterolo, G. Coco, L. Silvestro, and D. Operti. 2007. Evaluation of serum IgG4 antibodies specific to grass pollen allergen components in the follow up of allergic patients undergoing subcutaneous and sublingual immunotherapy. *Vaccine* 25: 957–964.
63. Golden, D. B., I. D. Lawrence, R. H. Hamilton, A. Kagey-Sobotka, M. D. Valentine, and L. M. Lichtenstein. 1992. Clinical correlation of the venom-specific IgG antibody level during maintenance venom immunotherapy. *J. Allergy Clin. Immunol.* 90: 386–393.
64. Wellmann, U., M. Letz, M. Herrmann, S. Angermüller, J. R. Kalden, and T. H. Winkler. 2005. The evolution of human anti-double-stranded DNA autoantibodies. *Proc. Natl. Acad. Sci. USA* 102: 9258–9263.
65. Zhang, J., A. M. Jacobi, T. Wang, and B. Diamond. 2008. Pathogenic autoantibodies in systemic lupus erythematosus are derived from both self-reactive and non-self-reactive B cells. *Mol. Med.* 14: 675–681.