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## 9G4+ auto-antibodies are an important source of apoptotic cell reactivity associated with high disease activity in systemic lupus erythematosus

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### Abstract

**Objective**—To determine the prevalence anti-apoptotic cell antibodies with the 9G4+ idiotype (9G4+) and the relationship between this reactivity and other known 9G4+ specificities and disease activity.

**Methods**—Sera from 60 SLE patients and 40 healthy control donors were incubated with apoptotic Jurkat cells and assayed for binding of 9G4+ antibodies by flow cytometry. These samples were also tested for 9G4+ reactivity against naïve B cells and total IgG and IgM anti-apoptotic cell antibody (AACA) reactivity.

**Results**—9G4+ antibodies bound late apoptotic cells in a majority of SLE patients (60%) but only 2 healthy control sera. Among samples with global IgM or IgG AACA, samples with 9G4+ AACA predominated. Patients with high levels of 9G4+ AACA were more likely to have active disease and this remained true even in patients with IgG AACA, or anti-dsDNA. Patients with lupus nephritis were also more likely to have 9G4+ AACA. While 9G4+ reactivity to apoptotic cells often coincided with anti-B cell reactivity, some samples had distinct anti-apoptotic cell or anti-B cell reactivity.

**Conclusion**—9G4+ antibodies represent a major species of anti-apoptotic cell antibodies in SLE serum and this autoreactivity is associated with disease activity. The anti-apoptotic cell reactivity of 9G4+ antibodies can be separated from the germline VH4-34 encoded anti-B cell autoreactivity. Our results indicate that apoptotic cells are an important antigenic source in SLE that positively select B cells with intrinsic autoreactivity against other self-antigens. This selection of 9G4+ B cells by apoptotic cells may represent an important step in disease progression.

During homeostasis billions of cells die through apoptosis daily and as a potential source of autoantigens these cells must be efficiently cleared in an immunologically silent fashion to

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prevent pathological autoimmune reactions (1). Defective clearance of apoptotic cells has been demonstrated both *in vitro* and *in vivo* in systemic lupus erythematosus (SLE), an autoimmune disease characterized by the generation of antibodies against multiple nuclear antigens (2-4). This is demonstrated by the high incidence of SLE in patients with genetic deficiency of C1q, a complement component involved in the opsonization and clearance of apoptotic cells. Like SLE patients, transgenic mice deficient in complement C1q develop autoantibodies or a lupus-like disease (5) and mice deficient in tyrosine receptor kinases necessary for apoptotic cell phagocytosis also develop severe autoimmunity (6). Furthermore, immunization of mice with apoptotic cells results in autoantibody production and autoimmune disease (7).

Anti-apoptotic cell antibodies (AACA) have been previously recognized in SLE serum and detected in lupus nephritis kidneys bound to glomerular apoptotic nucleosomes (8). SLE AACA can exercise pathogenic functions by promoting phagocytosis of apoptotic cells (9, 10), resulting in the engagement of intracellular TLR receptors, which leads to the release of type I IFN and other pro-inflammatory cytokines (11-13). While studies of IgG AACA in SLE have been focused on their influence on phagocytosis (3, 10, 14), systematic studies of their prevalence and significance are lacking. Similarly, the nature of IgG AACA and the processes leading to their generation and selection in SLE remain unclear. Of note, IgM AACA have been associated with protection against renal disease in SLE (15).

In this study, we systematically investigated the presence of IgG and IgM antibody binding to apoptotic cells in SLE patients using a flow cytometry-based assay and determined the contribution of antibodies bearing the 9G4 idiotype (9G4+) to this autoreactivity. The study of intrinsically autoreactive 9G4+ antibodies encoded by the VH4-34 gene is informative in SLE as these antibodies represent 10-40% of all serum IgG (16) due to defective germinal center censoring of VH4-34 B cells (17). The relevance of understanding the antigenic forces underpinning the expansion of 9G4+ antibodies in SLE is further illustrated by their high SLE specificity and their correlation with disease activity and specific clinical manifestations including lupus nephritis (18-21). Our results indicate that the presence of 9G4+ AACA is common in SLE and show that patients with elevated 9G4+ AACA are more likely to have active disease. These findings demonstrate that reactivity with apoptotic cell antigens contributes significantly to the expansion of a major autoreactive B cell population that is specifically expanded in SLE and provide the experimental basis for a better understanding of the antigenic forces involved in the pathogenesis of this disease.

## Methods

### Patient Samples and Study Design

Human serum samples were obtained from healthy donors (HCD) (n=40) and SLE patients (n=60). Adult male (1) and female (59) SLE study participants had at least 3 American College of Rheumatology criteria for SLE diagnosis. These patients had a wide range of clinical disease activity, as defined by SLEDAI scores (range 0-20, median SLEDAI 4). Patient age ranged from 18-85, with a mean age of 44. 57% of patients were Caucasian, 40% African American, and 3% Hispanic. An additional 25 SLE patients, 9 SLE patients with lupus nephritis, and 12 HCD were studied in a separate experiment. All samples were

obtained after informed consent, in accordance with protocols approved by the URMC or Emory University institutional review boards.

### Induction of cell death

The CD45-deficient human leukemic T cell line, Jurkat (J45.01), was maintained in complete medium in 5% CO<sub>2</sub> at 37°C. Apoptosis was induced by treatment with 20 μM camptothecin for 16-18 h. After blocking with normal mouse serum, apoptotic J45.01 cells were resuspended at 2.5 × 10<sup>6</sup> cells/mL and incubated with 25 μl serum, diluted with PBS to have equal amounts of IgM (0.4 mg/ml) or IgG (4 mg/ml), for 30 minutes at 4°C. Cells were then incubated with anti-human IgG-FITC and IgM-PE (BD Biosciences) or 9G4-FITC (custom conjugated, Southern Biotech) for 20 minutes at 4°C, washed, and stained with 7-aminoactinomycin D (7-AAD) (Southern Biotech) and in some experiments PE-Annexin V (Southern Biotech) before being analyzed by flow cytometry. Early apoptotic cells were defined as Annexin V+ 7-AAD-; late apoptotic cells as Annexin V+ and 7-AAD+. Binding for microscopy was done similarly except goat anti-rat Alexa Fluor 488 secondary antibody (Molecular Probes) was used to detect 9G4+ binding and the membrane permeable dye DRAQ5 (Axxora) was used as a nuclear stain. After staining, cells were plated into poly-D-lysine coated glass-bottom petri dishes (MatTek). B cell binding was measured on freshly obtained HCD peripheral blood lymphocytes as previously described (16).

### Flow cytometric analysis

Experiments were run on a LSR II 12-color system or a FACSCanto II 8-color system (BD). Imaging cytometry experiments were carried out on an ImageStream system (Amnis Corporation) and analyzed as previously described (22).

### Quantification of serum IgG and 9G4+ IgG

ELISA quantification of IgG, IgM, and 9G4+ IgG in all serum samples was carried out as described previously (16).

### Microscopy

Images were taken using an Olympus FV1000 Laser Scanning Confocal Microscope of the URSMD Confocal and Conventional Microscopy Core. A 60× objective (N.A. 1.42) was used to obtain high-resolution images within the linear range, and parameters were kept identical between the SLE and HCD sera incubated samples.

### Monoclonal antibody generation

Monoclonal antibodies were generated from single cell sorted memory B cells by PCR amplification of the immunoglobulin heavy and light chains, followed by expression vector cloning and transient transfection of 293T cells as described previously (23, 24).

### Data analysis and statistics

Flow cytometry data were analyzed with Flow-Jo (TreeStar) and all reported intensities are the median fluorescence intensity (MFI). GraphPad Prism version 5 was used for statistical tests. Correlation of non-parametric variables was determined using Spearman's rank

correlation. Odds ratios were analyzed by treating variables as dichotomous variables using the median value as the threshold value. For IgG AACA and IgM AACA, the mean plus two standard deviations of the HCD samples was used as the threshold. Active disease was defined as above the median SLEDAI of 4.

## Results

### SLE serum 9G4+ antibodies bind late apoptotic cells

Anti-apoptotic cell antibodies (AACA) have been reported in SLE with different and sometimes opposing functions (3, 10, 14). In addition, our preliminary results in SLE (25, 26) as well as studies of chronic lymphocytic leukemias (27) have previously demonstrated the ability of 9G4+ antibodies to bind apoptotic cells. In order to systematically study AACA in SLE and to understand the contribution and properties of 9G4+ AACA, we first established a flow cytometry assay to measure both 9G4+ AACA and global serum AACA of different isotypes. This assay, illustrated in Figure 1 for 9G4+ AACA, is based on the detection of antibody bound to Jurkat cells after camptothecin-induced apoptosis. These experiments were performed using healthy control serum or serum from SLE patients selected on the basis of elevated levels of serum 9G4+ antibodies as determined by ELISA (16). Specificity for antigens exposed on apoptotic cells but not on viable cells was established by documenting lack of reactivity with untreated cells. Given the demonstrated ability of 9G4+ antibodies to bind CD45 B220 glycoforms (16, 28), the CD45-deficient Jurkat clone J45.01 was used to avoid any global anti-lymphocyte reactivity.

The percentage of viable, early apoptotic, and late apoptotic cells was similar after incubation with SLE or HCD serum, demonstrating that under these conditions SLE serum was not cytotoxic (Figure 1A). 9G4 staining of early apoptotic cells was low and did not differ between HCD and SLE serum. In contrast, 9G4 staining was much higher in late apoptotic cells incubated with SLE serum but not HCD serum. This observation was confirmed using flow based single cell microscopy. Utilizing combined 7-AAD and DRAQ5 staining, Amnis ImageStream analysis provides more accurate morphometric separation of live, early apoptotic, and late apoptotic populations and allows direct visualization of apoptotic bodies and localization of nuclear material (Figure 1B-C) (22). This approach demonstrated that 9G4+ antibodies strongly bind to 7-AAD+ late apoptotic cells and more weakly to apoptotic bodies but not live or early apoptotic cells (Figure 1D). Additionally, small DRAQ5+ particles consisting primarily of nucleic acids did not have 9G4+ staining.

### 9G4+ anti-apoptotic cell antibodies are common in SLE

The frequency of 9G4+ AACA was ascertained in serum samples obtained from a group of 60 unselected SLE patients and 9 HCD using flow cytometry of camptothecin-treated Jurkat cells. Consistent with the results described above, only 7-AAD+ late apoptotic cells incubated with SLE serum had bound 9G4+ antibodies; 7-AAD- or cells incubated with HCD serum did not (Figure 2A). When defined as having MFI greater than the mean MFI of the HCD plus two standard deviations ( $MFI > 328$ ), positive 9G4+ AACA binding was present in 60% of SLE patients with varying degrees of reactivity (Figure 2B). One HCD sample showed a low level of 9G4+ AACA at this threshold ( $MFI = 331$ ). These results were

validated in a larger group of 31 HCD in which 1 sample had 9G4+ AACA (data not shown). This frequency is consistent with the observed occurrence of autoreactivity in the general population (29).

We next investigated the correlation between the levels of total 9G4+ antibodies, which we previously reported to account for 10%-40% of all SLE serum IgG (17), and the MFI of 9G4+ AACA binding. Levels of serum 9G4+ IgG antibodies above 0.2 mg/ml were present in 75% of patients, and 9G4 IgG titers correlated with the MFI of 9G4+ AACA ( $p=0.014$ ,  $r=0.313$ ) (Figure 2C). However, approximately one third of patients with elevated 9G4+ IgG levels did not demonstrate anti-apoptotic cell reactivity, indicating that binding to apoptotic cells is not an intrinsic, universal property of antibodies expressing the 9G4+ idiotype encoded by the germline VH4-34 heavy chain. Rather, our data show that this property is present in only a fraction of 9G4+ antibodies that appear to be selected for in a significant portion of the SLE population.

### **A large majority of samples with global IgM or IgG AACA have 9G4+ AACA**

To better understand the participation of 9G4+ antibodies in the anti-apoptotic cell response in SLE, we determined the overall AACA binding of total IgM and IgG antibodies. AACA of both isotypes were detected in SLE sera with 32% of samples exhibiting IgM AACA and 41% IgG AACA (Figure 3A). In contrast, both isotypes were rare in HCD; in a large group of HCD, 10% had low IgG AACA and 3% had IgM (N=31; not shown). In the SLE samples with IgM or IgG AACA, samples with 9G4+ AACA predominated, making up over two thirds (71%) of the samples (Figure 3A). Furthermore, sera with 9G4+ AACA had the highest levels of global IgM or IgG AACA. Thus, when samples with IgG AACA were ranked by IgG MFI, 90% of the top third of samples also had 9G4+ AACA and the top third of samples with IgM AACA all had 9G4+ AACA. When IgM and IgG AACA were analyzed together, 22% of samples had both IgM and IgG activity (Figure 3B). The distribution of IgG and IgM differed between samples with or without 9G4+ AACA (chi-square,  $p=0.008$ ). Samples with 9G4+ AACA were more likely to have IgM AACA and in particular IgM and IgG AACA together than samples without (Figure 3C). In fact, no samples that lacked 9G4+ AACA had AACA of both isotypes.

Some samples with 9G4+ AACA had neither IgM nor IgG AACA. This discrepancy could be explained by differences in the detection sensitivity of the assays utilized or may indicate the presence of IgA 9G4+ AACA. The latter possibility is supported by a separate analysis of SLE 9G4+ IgA monoclonal antibodies performed in our laboratory that demonstrated significant anti-apoptotic cell activity among 9G4+ IgA antibodies (24).

### **Apoptotic cell binding and B cell reactivity of 9G4+ antibodies segregate in some SLE patients**

As shown in Figure 2, some SLE patients had no anti-apoptotic cell reactivity despite having high levels of serum 9G4+ IgG antibodies. 9G4+ antibodies also react with naïve B cells (B cell binding; BCB) through their ability to bind surface B220, and this reactivity is common in patients with SLE (16). Therefore, we determined the relationship between these two types of 9G4+ autoreactivity in the same cohort of SLE patients. While apoptotic cell and B

cell specificities were simultaneously present in many samples, other samples had only one or the other (Figure 4A). Because of this, 9G4+ AACA and 9G4+ BCB were not correlated. Of the samples with 9G4+ apoptotic or B cell reactivity, 25% had only apoptotic cell binding and a few samples (12%) had only B cell reactivity (Figure 4B). Serum is a mixture of antibodies with differing specificities and samples with both specificities could consist of a mixture apoptotic and B cell reactivities rather than antibodies with shared specificity against both targets. To clarify how individual antibodies contributed to B cell and apoptotic reactivities, we generated recombinant 9G4+ monoclonal antibodies from single cell sorted memory B cells (IgD-CD27+) from an SLE patient. As illustrated by representative examples in Figure 4C, serum binding profiles (either apoptotic cell binding, BCB or both), are recapitulated by 9G4+ monoclonal antibodies. These distinct autoreactivity profiles have been confirmed with a large panel of monoclonal antibodies whose binding properties and structural correlates will be published separately (24).

### **9G4+ apoptotic cell binding is associated with high disease activity, lupus nephritis, and anti-dsDNA antibodies**

To evaluate disease correlates and to begin understanding pathological implications, we determined the association between disease activity, as measured by SLEDAI, and the MFI of 9G4+ AACA binding. We also determined the association between SLEDAI and concentration of 9G4+ IgG, 9G4+ BCB antibodies, IgG AACA and anti-dsDNA antibodies. The MFI of 9G4+ AACA was positively correlated with disease activity ( $r=0.322$ ,  $p=0.012$ ) (Figure 5A). In contrast, 9G4+ binding to B cells was only weakly correlated with SLEDAI ( $r=0.201$ ,  $p=0.123$ , data not shown). When 9G4+ AACA and SLEDAI were analyzed as dichotomous variables, patients with high 9G4+ AACA binding were more likely to exhibit active disease than those with low 9G4+ AACA (odds ratio (OR) =4.76,  $p=0.008$ ). As previously reported, serum 9G4+ IgG concentration was also positively correlated with SLEDAI ( $r=0.266$ ,  $p=0.041$ ) (Figure 5B). However, almost all samples (85%) with high serum 9G4+ IgG concentration and active disease also had high 9G4+ AACA. In the group of patients with high serum 9G4+ IgG, patients that also had high 9G4+ AACA were more likely to have active disease (OR=7.7,  $p=0.032$ ) than those with low 9G4+ AACA. Because of the clinical significance of lupus nephritis (LN) and its association with high serum 9G4+ antibodies (20), we also examined 9G4+ AACA in SLE patients with LN. 7 of the 60 SLE patients had LN and in a separate experiment we recruited an additional 9 LN patients. We found, 9G4+ AACA were much more prevalent in patients with lupus nephritis (88%) than those without (57%) ( $p=0.0212$ , Fisher's exact test). 9G4+ AACA were found both in patients with active nephritis and stable disease, however, active patients had some of the highest levels of 9G4+ AACA and the two LN patients without 9G4+ AACA were both stable.

Coinciding anti-dsDNA antibody titers were available for 37 patients. Anti-dsDNA titers were highly correlated with 9G4+ AACA MFI ( $r=0.759$ ,  $p<0.001$ ) (Figure 5C). In agreement with previous studies (30), serum anti-dsDNA titers correlated with disease activity ( $r=0.433$ ,  $p=0.008$ ). However, patients with both high 9G4+ AACA and anti-dsDNA antibodies were more likely to have active disease (80%, OR=9.5,  $p=0.012$ ) than patients grouped based only on anti-dsDNA (67%, OR=7.5,  $p=0.007$ ).

Like 9G4+ AACA, IgG AACA was correlated with disease activity, but to a lower degree, ( $r=0.271$ ,  $p=0.034$ ) (Figure 5C). Patients with IgG AACA positive serum that also had 9G4+ AACA were much more likely to have active disease (75%) than those without 9G4+ AACA (25%) (OR= 9,  $p=0.020$ ) (Figure 5D). IgM AACA were neither negatively nor positively correlated with SLEDAI and were not protective in patients with IgG AACA. Patients with both IgM AACA and IgG AACA were actually more likely to have active disease than those with IgG AACA alone (62% vs. 43%), although this difference was not statistically significant.

### 9G4+ antibody binding to apoptotic cells localizes to the perinuclear area

Several distinct antigens including oxidized lipids (31), nucleic acids (9), ribonucleoproteins (32), and nucleosomes (33) have been defined as targets of anti-apoptotic antibodies. During apoptosis, these antigens cluster in apoptotic blebs on the cell surface (34). The regions of Jurkat apoptotic cells recognized by 9G4+ AACA were examined by confocal microscopy from two different SLE samples with different AACA binding strength (intermediate: SLE1; and strong: SLE2). In contrast to HCD sera, which did not generate significant staining, strong 9G4+ antibody staining was clearly detected with both SLE samples (Figure 6) and two different staining patterns were observed. While 9G4 staining was predominantly perinuclear in both cases, SLE1 serum created a spotted pattern with small dots outlining the nucleus (Figure 6A), SLE2 displayed 9G4 staining in a much more homogeneous perinuclear ring pattern (Figure 6B). 9G4+ antibodies also clearly bound apoptotic blebs in this sample. Consistent with the morphometric analysis described in Figure 1, only limited binding to small apoptotic bodies was observed, many particles consisting of nucleic acid had no 9G4 staining. Because 9G4+ antibodies did not colocalize with nucleic acids we examined association between 9G4+ AACA and other common SLE auto-reactivities in a separate group of 25 patients. We found that patients with high 9G4 AACA were more likely to have anti-RNP, anti-Smith, and anti-Ro. The difference in anti-Ro was most evident and was statistically significant (63% vs 21%,  $p= 0.042$ , Fisher's exact test).

## Discussion

Anti-nuclear antibodies are not uncommon in otherwise healthy individuals (29), demonstrating that a breakdown in tolerance is insufficient for autoimmune disease and tolerance breakdown alone can not explain why a patient develops a specific autoimmune disease. Instead, the selection by disease-specific self-antigens is an important factor in determining how autoimmunity manifests. These considerations are of central significance for SLE, a systemic autoimmune disease characterized by multiple autoantibodies, some of which (anti-dsDNA, anti-Smith and anti-ribosomal P antibodies) are highly specific for SLE. AACA constitute another relevant autoantibody system, as SLE is characterized by defective clearance of apoptotic cells, which express a high density of SLE immunogens. AACA can mediate multiple pathogenic mechanisms, including tolerance breakdown, epitope spreading and further induction of autoantibodies capable of both amplifying inflammation and directly inducing tissue damage (8, 12, 35-37). Elevated levels of 9G4+ antibodies are also specific for SLE and are prevalent in active SLE patients (16). The canonical intrinsic autoreactivity of 9G4+ antibodies is imparted by a germline-encoded hydrophobic patch that

mediates binding to N-acetyl-lactosamine (NAL) sugar and accounts for the striking anti-B cell autoreactivity of 9G4+ antibodies *in vivo* and *in vitro* (16). NAL sugar chains have been shown to be exposed on the surface of apoptotic cells (38, 39), and furthermore, our preliminary results (25, 26) indicated the potential of 9G4+ antibodies to bind apoptotic cells. Additionally, defective censoring leads to the accumulation of 9G4+ B cells in SLE germinal centers (17), a site of accumulation of uncleared apoptotic cells (4).

In this work we tested the hypothesis that defective tolerance and the localization of 9G4+ B cells in germinal centers would result in selection of 9G4+ antibodies with AACA reactivity that would contribute the global AACA response in SLE. The results presented here support this hypothesis. Elevated 9G4+ AACA are present in a majority of unselected SLE patients (60%). Moreover, 9G4+ AACA MFI correlates with the degree of disease activity measured by the SLEDAI, and patients with high 9G4+ AACA were significantly more likely to have active disease and LN. While concentrations of IgG AACA, serum 9G4+ IgG, and anti-dsDNA antibodies also correlated with disease activity, patients with high values for these correlates that also had high levels of 9G4+ AACA were more likely to have active disease than those with lower levels of 9G4+ AACA. Combined, our results identify 9G4+ AACA as an important marker of disease activity and possibly of disease severity, an intriguing finding whose clinical implication need to be conclusively validated by ongoing longitudinal studies.

During normal homeostasis, the phagocytosis of apoptotic cells is generally anti-inflammatory (40). This tolerogenic anti-inflammatory response is altered in SLE patients because of both changes in SLE monocytes (41) and a shift to Fc receptor internalization through AACA (42). SLE AACA alone are sufficient to promote inflammation, as HCD monocytes incubated with necrotic cell material and phagocytosis-promoting SLE antibodies produce large amounts of inflammatory cytokines (12). The abundance of 9G4+ antibodies in SLE means 9G4+ AACA are likely an important part of this process. Additionally, because 9G4+ antibodies can recognize a diverse array of self-antigens (24, 43), 9G4+ B cells presenting apoptotic cell derived antigens may result in epitope spreading that expands and perpetuates anti-apoptotic cell responses and enhance antigen presentation of apoptotic cell derived T cell epitopes such histone peptides from nucleosomes (44).

9G4+ AACA and anti-dsDNA correlated with one another and with disease activity but were nonetheless dissociated in a significant fraction of patients. This indicates that these two autoantibody species, while often produced concurrently by active patients, recognize separate antigens. This conclusion is further supported by the co-localization results provided by ImageStream and confocal microscopy studies (Figures 1 and 5, respectively), in which 9G4+ antibody binding to apoptotic cells did not merge with nucleic acid staining and many DRAQ5 staining nuclear bodies had no associated 9G4 staining. This suggests that 9G4+ antibody binding to apoptotic cells is not mediated by the ability of a fraction of these serum antibodies to recognize DNA. Although it is only correlative, the higher incidence of anti-Ro in patients with 9G4+ AACA is consistent with our microscopy findings, as Ro localizes to apoptotic blebs (45) and to the peri-nuclear space during apoptosis (46).



While the precise antigenic targets recognized by 9G4+ antibodies in apoptotic cells remains to be determined, their reactivity with other relevant antigens and established structure-function correlations provide important clues. Since the expression of the 9G4+ idiotype, NAL binding, and BCB activity are all dependent on the conservation of the VH4-34 germline-encoded framework-1 hydrophobic patch (24, 47), potentially the AACA reactivity of 9G4+ antibodies could also have similar structural requirements and would therefore segregate with BCB activity. This model would point to recognition of antigens shared between the two cellular targets, in particular NAL. Several lines of evidence, however, argue against this possibility. 9G4+ AACA were observed in the absence of BCB activity in up to 23% of patients and a smaller fraction of patients displayed only 9G4+ BCB. Moreover, separate studies of monoclonal antibodies indicate that the apoptotic and B cell reactivities of 9G4+ antibodies are dependent on different regions of the VH4-34 heavy chain and are likely determined by distinct antigens on the corresponding cellular targets (24). That 9G4+ AACA reactivity is not largely due to the canonical anti-NAL binding is also supported by the observation that 9G4+ antibodies in sera from HIV patients bind to B cells (48) but not to apoptotic cells (unpublished observation). It is possible that 9G4+ AACA could originate from 9G4+ B cells initially recognizing canonical NAL antigens on B cells, red blood cells, or other tissues that subsequently are diversified by somatic hypermutation and selection by apoptotic cells. Yet, given the continuous presence of B cells that could exert selective pressure in the germinal centers, this model would also predict the persistence of 9G4+ BCB autoreactivity concurrently with the development of AACA reactivity. Our results are more consistent with independent triggering and selection of different 9G4+ B cell clones by either B cells or apoptotic cells with the provision that the retention of the VH4-34 germline FR1 hydrophobic patch would endow most apoptotic cell-reactive 9G4+ antibodies with BCB activity as well. Irrespective of the molecular underpinnings and temporal events of selection, these results demonstrate that like other lupus autoantibodies, while a significant fraction of lupus patients break tolerance against apoptotic cell antigens, this tolerance breakdown is not a universal phenomenon in SLE. Approximately 20% of patients with elevated 9G4+ IgG levels did not demonstrate AACA reactivity, indicating that binding to apoptotic cells is not an intrinsic property of antibodies expressing the 9G4+ idiotype. Rather, this property is present in only some 9G4+ antibodies that appear to be selected in a segment of the SLE population.

Combined, the data presented here strongly suggest that measurement of AACA in general and of 9G4+ AACA in particular provide a useful tool to assess disease activity in SLE. Moreover, the presence of these antibodies may provide a way to segment lupus patients with different genetic and immunological defects. Thus, a better understanding of the origin and consequences 9G4+ antibodies against apoptotic cells has the potential to increase our understanding of SLE pathogenesis and provide new approaches to treatment.

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## Abbreviations

<b>7AA-D</b>	7-aminoactinomycin D
<b>AACA</b>	Anti-apoptotic cell antibody
<b>BCB</b>	B cell binding
<b>HCD</b>	Healthy control donor
<b>LN</b>	lupus nephritis
<b>MFI</b>	Median fluorescence intensity
<b>NAL</b>	N-acetyl-lactosamine
<b>OR</b>	Odds ratio

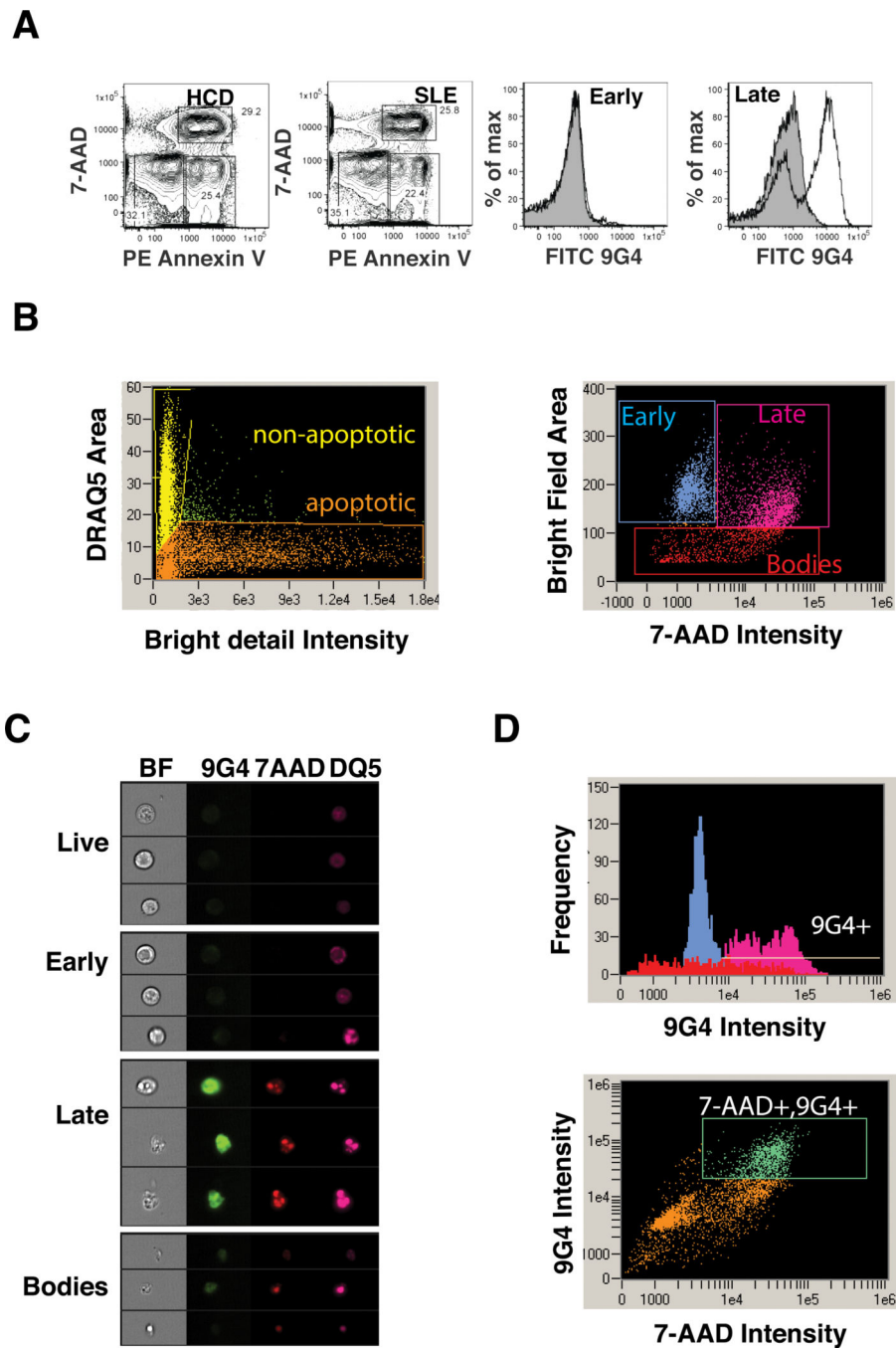
<b>SLE</b>	Systemic lupus erythematosus
<b>SLEDAI</b>	Systemic Lupus Erythematosus Disease Activity Index

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**Figure 1.** 9G4+ antibodies in SLE serum bind late apoptotic cells and apoptotic bodies. **A**, After incubation with HCD or SLE annexin V and 7-AAD were used to divide cells into live (Annexin V- 7-AAD-), early apoptotic (Annexin V+ 7-AAD-) or late apoptotic (Annexin V+ 7-AAD+) populations. 9G4 histograms are shown for the early apoptotic and late apoptotic populations. HCD serum treated cells are indicated by the shaded histograms and SLE serum treated cells by the unshaded histograms. **B**, Plotting nuclear size (DRAQ5 area) vs. nuclear fragmentation (DRAQ5 bright detail intensity) separates treated Jurkat cells into

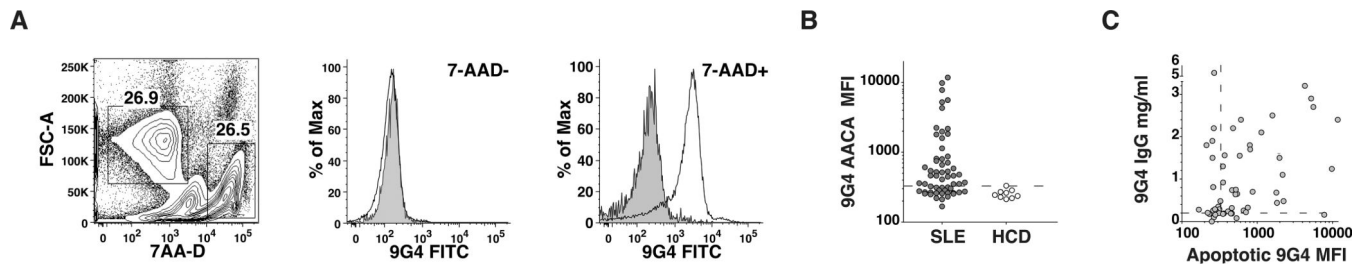
live (yellow) and apoptotic cells (orange) based on nuclear morphology. Apoptotic cells can be further separated into early apoptotic cells (blue), late apoptotic cells (magenta), and apoptotic bodies (red) based on membrane integrity (7-AAD intensity) and cell size (bright field area). C, Example images of each population showing bright field, 9G4+ binding (green), 7-AAD (Red), and DRAQ5 (magenta). D, Only 7-AAD+ cells stain brightly for 9G4+ providing further evidence that 9G4+ AACA bind to late apoptotic cells (magenta) and some apoptotic bodies (red), but not early apoptotic cells (blue).

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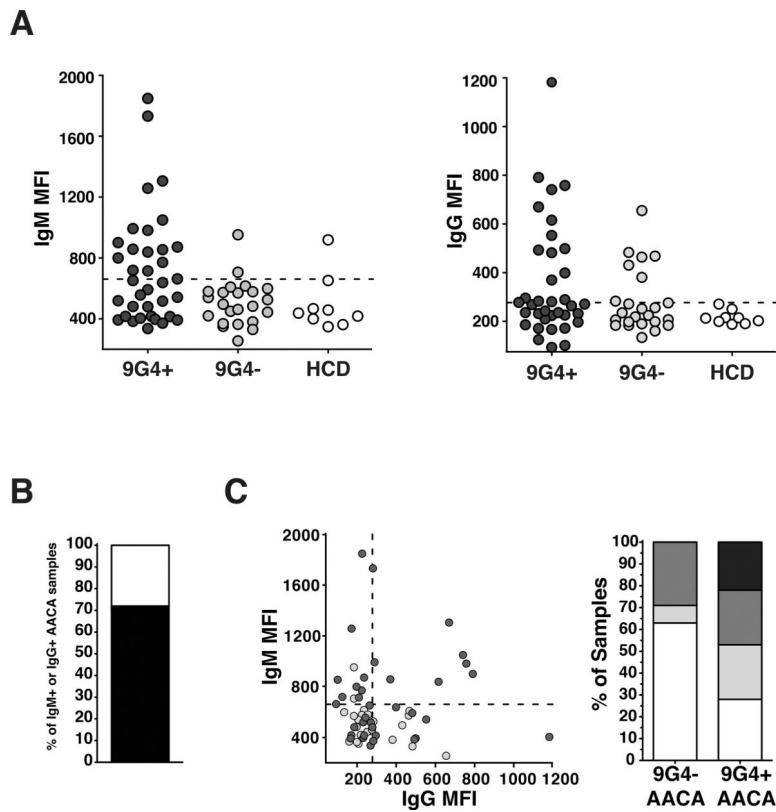
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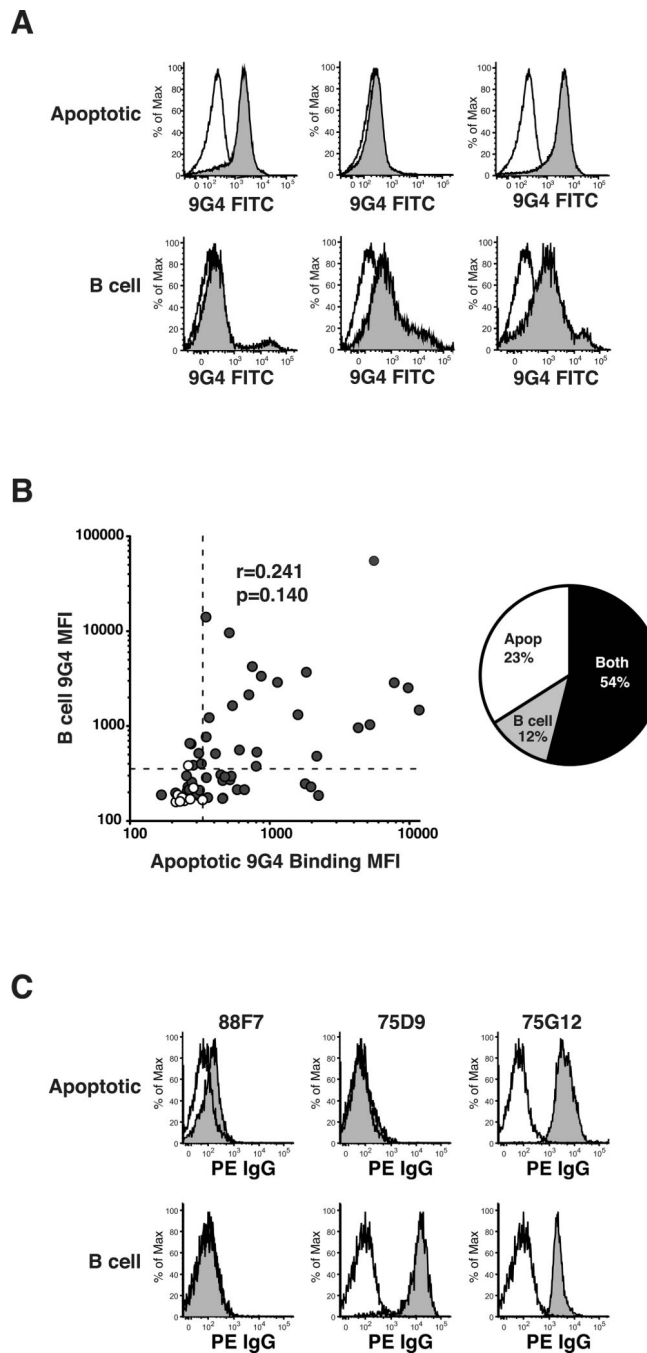
**Figure 2.**

9G4+ AACA are common in SLE patients but rare in HCD. **A**, Representative histograms from late apoptotic (low FSC, high 7-AAD) or viable (high FSC, low 7-AAD) Jurkat cells after incubation with serum from HCD (shaded) or SLE (open) donors. **B**, Plot of 9G4+ AACA MFI after incubation with SLE (filled circles) or HCD (open circles) serum. The positive cutoff based on the HCD MFI is indicated by the dotted line. **C**, Serum 9G4+ IgG concentration of SLE samples correlates with 9G4+ AACA, but not all samples with high serum 9G4+ IgG levels have high 9G4+ AACA.





**Figure 3.** Samples with 9G4+ AACA constitute a large majority of samples with global IgM and IgG AACA. **A**, IgM or IgG staining of 7-AAD+ Jurkat cells incubated with SLE serum with (dark grey) or without (light grey) 9G4+ AACA or HCD (white) serum. **B**, The bar graph shows the percentage of IgM+ and/or IgG+ samples with AACA that did (filled) or did not (open) have 9G4+ AACA. **C**, The relationship between IgG, IgM, and 9G4+ AACA MFI is shown for SLE samples with (dark grey circles) or without (light grey circles) 9G4+ AACA. The bar graph on the right shows the percentage of samples with neither IgM nor IgG AACA (white), only IgM AACA (light grey), only IgG AACA (dark grey), or both IgM and IgG (black) for samples with or without 9G4+ AACA.



**Figure 4.** 9G4+ apoptotic and B cell binding can be distinct in SLE serum. **A**, Representative histograms of apoptotic (top) or naïve B cell (bottom) staining after incubation with one HCD serum (open) or 3 different SLE sera (filled). **B**, 9G4+ MFI plotted for naïve B cells (IgD+ CD27-) and apoptotic cells incubated with HCD (open circles) or SLE (filled circles) serum, the positive threshold for each parameter is indicated by a dotted line. The percentage of each type of 9G4+ binding for the samples with reactivity is shown on the right. **C**, Monoclonal antibody IgG apoptotic (top) or naïve B cell (bottom) staining. 152B5, an

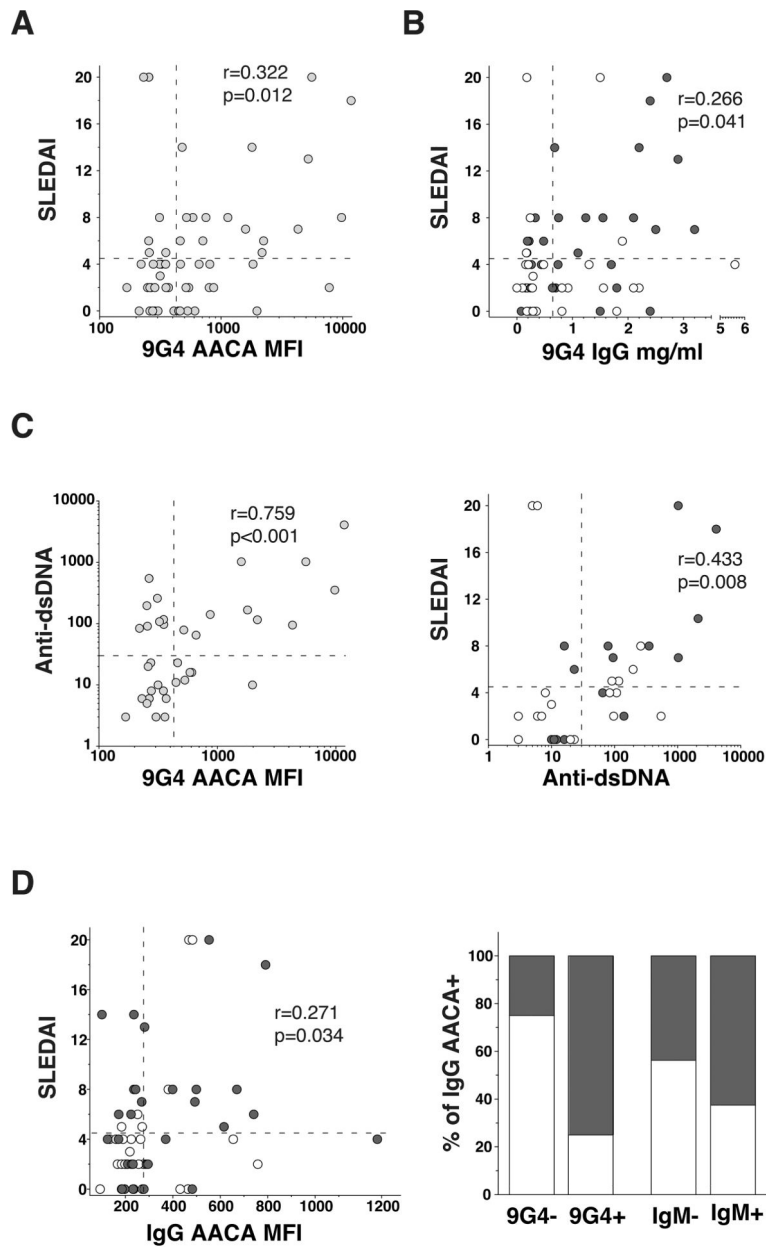
antibody negative for both specificities is shown in the open histogram and the indicated monoclonal in the filled histogram.

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**Figure 5.** SLE patients with high 9G4+ AACA are more likely to have active disease. In all plots except D, the dotted lines indicate the median value of the measured parameter or a SLEDAI value greater than the median SLEDAI of 4, in D the dotted line on the X axis indicates the positive threshold value; dark grey and white indicate samples with and without 9G4+ AACA, respectively. **A**, 9G4+ AACA MFI plotted against SLEDAI. **B**, The concentration of 9G4+ IgG plotted against SLEDAI for samples with or without 9G4+ AACA. **C**, 9G4+ AACA MFI plotted against anti-dsDNA titer. To the right is anti-dsDNA plotted against

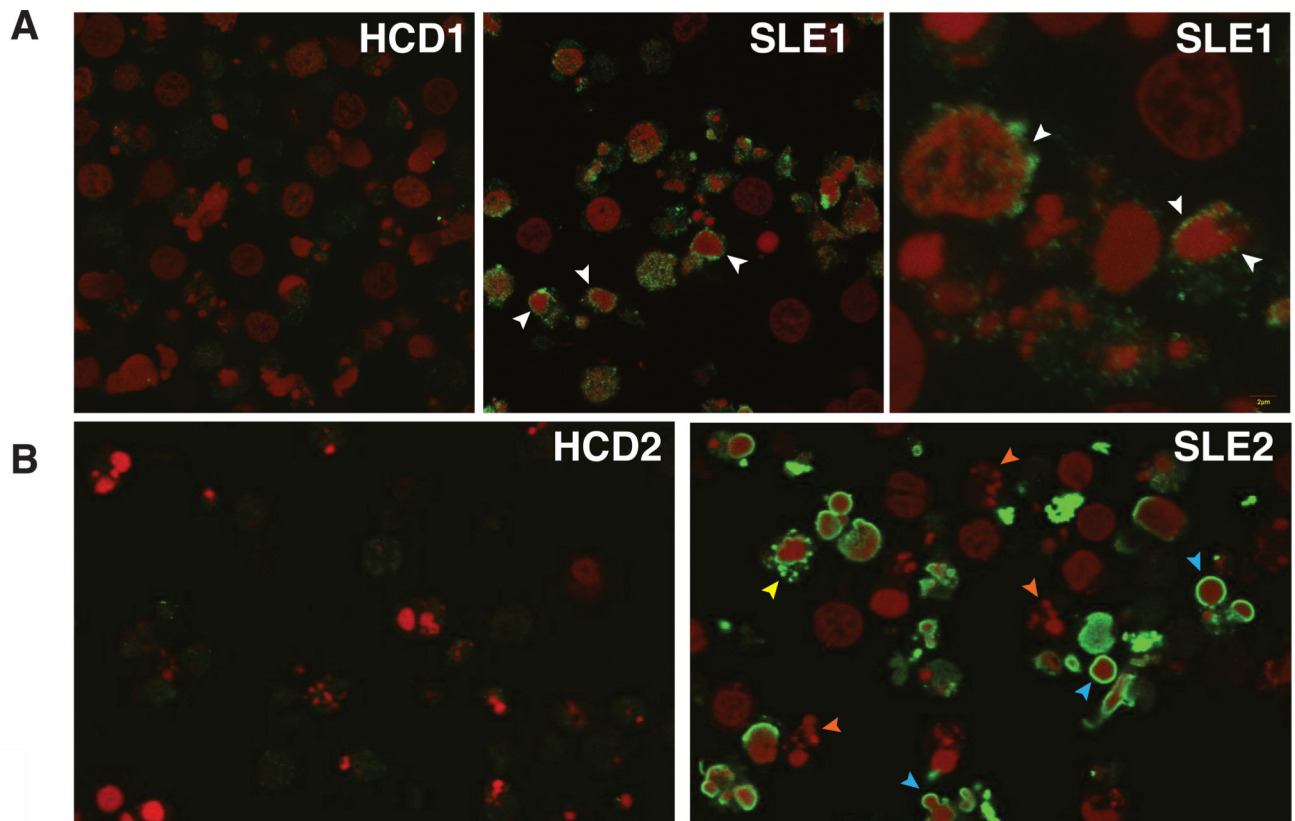
SLEDAI for samples with and without 9G4+ AACA. **D**, IgG AACA plotted against SLEDAI for samples with and without 9G4+ AACA. To the right is plotted the percentage of patients with IgG AACA that have active disease (filled portion of the bar) of patients with or without 9G4+ AACA or with or without IgM AACA.

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**Figure 6.** 9G4+ antibodies from SLE patients localize to the perinuclear space and apoptotic blebs. Confocal microscopy of apoptotic Jurkat cells incubated with HCD (left) or SLE (right) serum and stained with 9G4+ (green) and the nuclear stain DRAQ5 (red). **A**, SLE1 9G4+ staining is localized to perinuclear spots (white arrows); the left most panel shows these perinuclear spots digitally enlarged. **B**, SLE2 incubated cells have 9G4+ localized to a thick perinuclear rings (blue arrows) and to apoptotic blebs (yellow arrows); some nuclear fragments have no associated 9G4+ staining (orange arrows).