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Blebs and Apoptotic Bodies Are B Cell Autoantigens

Brian A. Cocca,* Amy M. Cline,* and Marko Z. Radic2*†

Mounting evidence suggests that systemic lupus erythematosus autoantigens are derived from apoptotic cells. To characterize the potential interactions between apoptotic cells and B cells, the D56R/S76R variant of 3H9, a murine autoantibody that binds to DNA, chromatin, and anionic phospholipids, was compared with DNA4/1, a human anti-DNA autoantibody. Flow cytometry revealed that only D56R/S76R bound to Jurkat cells treated with either of three distinct proapoptotic stimuli, Ab binding was dependent on caspase activity, and immunoreactivity developed subsequent to annexin V binding. Confocal microscopy established a structural basis for the distinct kinetics of binding. D56R/S76R preferentially bound to membrane blebs of apoptotic cells, whereas annexin V binding did not require blebs. Inhibition of ROCK I kinase, an enzyme that stimulates nuclear fragmentation and fragment distribution into blebs, significantly reduced Ab binding. Because members of the collectin and pentraxin families of serum proteins bind to blebs on apoptotic cells and assist in the clearance of cellular remains, our results suggest that Abs to blebs could affect the recognition of apoptotic cells by cells of the innate immune system and thus modify tolerance to nuclear Ags. The Journal of Immunology, 2002, 169: 159–166.

If autoantigens are derived from apoptotic cells, then a disruption of the normal pathways of apoptotic cell recognition and clearance may result in autoimmunity. This expectation is supported by experimental evidence. Inherited deficiencies in the recognition of apoptotic cells coincide with a delayed clearance of cell remnants and an increased incidence of autoimmune responses. Thus, characteristic autoantibodies and lupus-like pathology arise in individuals lacking the complement protein C1q (16), a protein that binds apoptotic cells. Similarly, mice with deficiencies in C1q (17) or the serum amyloid P (SAP) component (18) produce autoantibodies and manifest autoimmune disease. Both C1q and SAP are among a category of molecules capable of recognizing patterns of negative charge that are characteristic of apoptotic cells (19, 20). Therefore, the impaired recognition of surface features of apoptotic cells may be linked to an increased probability of autoimmune response.

Defects in the phagocytosis of apoptotic cells may also lead to the production of autoantibodies, although it has been difficult to demonstrate that the absence of a single receptor for binding to apoptotic cells leads to pathogenesis (21). Presumably, this difficulty arises because phagocytes have a variety of receptors that can mediate the recognition and uptake of apoptotic cells (22, 23). Despite this apparent redundancy, Scott et al. (24) uncovered a direct link between the delayed clearance of apoptotic cells and the induction of autoimmunity. These researchers identified a receptor tyrosine kinase, Mer, whose activity is required for phagocytosis of apoptotic cells. In mice with a deletion of the Mer catalytic domain, clearance of apoptotic cells is delayed and anti-DNA autoantibodies are produced (24). Lu and Lemke (25) emphatically extended these observations by combining Mer deficiency with the deletions of Axl and Tyro 3, two additional members of the Mer kinase family. Triply-deficient mice exhibit a striking constellation of autoimmune phenomena, including the production of autoantibodies to DNA and phospholipids and the activation of lymphoid and myeloid cell populations (25). Previous experiments showed that murine autoantibodies can recognize apoptotic cells (26–28) and that an experimentally induced excess of apoptotic cells can induce the production of anti-nuclear and anti-phospholipid autoantibodies (29–31). Although some Abs to apoptotic cells may contribute to the clearance of cell debris as part of normal cell disposal mechanisms (32), other Abs to apoptotic cells likely participate in the...
pathogenesis of autoimmune disease (33). Collectively, these studies suggest that defects in the clearance of apoptotic cells or an excessive load of cell remnants increase the risk of an autoimmune response.

Nevertheless, the hypothesis that apoptotic cells provide nuclear autoantigens raises important questions: Given the rapid and efficient uptake of apoptotic cells by phagocytes, how could cells of the adaptive immune system compete for access to apoptotic cells? Could unique features of apoptotic cells stimulate or inhibit adaptive immune responses? What receptors could B cells use to bind apoptotic cells and what would be the results of such encounters? A more thorough understanding of interactions between Ig receptors and the surface of apoptotic cells may provide answers to these questions.

Previously, we used site-directed mutagenesis to evaluate the role of somatic mutations in the binding of autoantibodies to a complex between phosphatidylserine, a phospholipid that is externalized by apoptotic cells, and $\beta_2$ glycoprotein I ($\beta_2$GPI), a serum protein that binds to phosphatidylserine on the surface of apoptotic cells (34). The starting point for our studies was 3H9, a murine autoantibody reactive with DNA, chromatin, and negatively charged phospholipids (35). We observed that binding to the phosphatidylserine-$\beta_2$GPI complex may be enhanced by somatic mutations in the $H$ chain of 3H9 and that additional increases in affinity are achieved by introduction of arginines at sites that sustain such mutations in vivo. In addition, two variants of 3H9, the increased affinity mutant D56R/S76R and the germline revertant R53G/I57T/D65G, demonstrated binding to apoptotic cells.

Positive selection for mutations in 3H9 and other Abs that recognize a complex between phosphatidylserine and $\beta_2$GPI implies direct contacts between B cells and apoptotic cells. To explore the mechanism of Ab binding to apoptotic cells, we used D56R/S76R and compared its binding to DNA4/1, a human anti-DNA autoantibody (36). Confocal microscopy revealed that only D56R/S76R preferentially interacts with blebs on the apoptotic cell surface. Analogous interactions between B cells and apoptotic cells may contribute to the regulation of tolerance to nuclear Ags.

Materials and Methods

Construction and expression of single-chain Fv

The D56R/S76R (37) and DNA4/1 single-chain variable fragments (scFv) (36) were described previously. The complete scFv fusion proteins included the c-Jun leucine zipper, the protein A "B" domain, and a penta-histidine tag, as was confirmed by DNA sequencing of the two expression vectors. The scFv were purified as described elsewhere (37). Briefly, soluble scFv were recovered from the periplasm by digestion of the bacterial cell wall with lysozyme, dialyzed overnight against binding buffer (50 mM Tris-HCl, pH 8.0, 1.0 M NaCl, 40 mM imidazole, 0.5% Tween 20). The purified scFv were eluted with 2.0 ml of elution buffer (50 mM Tris-HCl, pH 8.0, 1.0 M NaCl, and 350 mM imidazole), dialyzed overnight against PBS, and analyzed by SDS-PAGE and Coomassie blue staining.

Cell culture and induction of apoptosis

Jurkat cells were harvested from culture and resuspended at a density of 10^6/ml in RPMI 1640 containing 10% FBS. Apoptosis was induced for 12 h with 1.0 $\mu$M staurosporine (Sigma-Aldrich, St. Louis, MO), 2.0 $\mu$M camptothecin (Sigma-Aldrich), or 200 ng/ml anti-Fas mAb (clone 7C11; Beckman Coulter, Brea, CA). To inhibit apoptosis, parallel cultures were preincubated for 2 h with 20 $\mu$M Z-Val-Ala-Asp(Ome)-fluoromethylketone (Z-VAL-OMe)-fmk (Enzyme System Products, Livermore, CA). At the end of the apoptosis induction period, 5 $\times$ 10^6 cells were aliquoted into tubes and stained for flow cytometry or confocal microscopy as described below.

Flow cytometry

Cells were washed with 4.0 ml of ice-cold HBBS (Mediatech, Herndon, VA) containing 1.0 mM CaCl$_2$, 3% FBS, and 0.02% NaN$_3$ (HBBS/FBS). Washed cells were incubated with 10 $\mu$g/ml D56R/S76R or DNA4/1 scFv for 15 min on ice, washed twice as above, and stained with FITC-conjugated annexin V (BD Biosciences, San Diego, CA) and alkaline-phococyanin-conjugated rabbit IgG (Molecular Probes, Eugene, OR), as recommended by the manufacturer. The rabbit IgG binds to the protein A domain of the scFv fusion protein (37). Thirty thousand events were examined per sample on a FACS Calibur (BD Biosciences). The staining profiles were analyzed using FlowJo software (Treestar, San Carlos, CA).

Confocal microscopy

Cells were washed with HBBS and fixed with ice-cold 4% paraformaldehyde for 10 min. Fixed cells were washed with HBBS/FBS and incubated with 10 $\mu$g/ml D56R/S76R or DNA4/1 for 15 min on ice. After incubation with the scFv, cells were washed once with HBBS/FBS and incubated with biotinylated annexin V (BD Biosciences) for 15 min on ice. Cells were washed twice with HBBS/FBS and stained with streptavidin-conjugated Alexafluor 488 (Molecular Probes), Rhodamine red-conjugated human serum IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and the nucleic acid dye TO-PRO-3 (Molecular Probes). After a 15-min incubation on ice, cells were washed with HBBS/FBS and mounted onto poly-l-lysine-coated glass slides for viewing with a LSM 510 laser scanning microscope (Zeiss, Thornwood, NY).

ROCK I kinase inhibition

To inhibit membrane blebbing, we used the E6-1 subclone of Jurkat T cells (American Type Culture Collection, Manassas, VA) that is highly sensitive to Fas ligation. Cells were incubated with 50 $\mu$g/ml Y-27632 (Tocris, Ballwin, MO), a specific inhibitor of ROCK I kinase, 1 h before the addition of 50 ng/ml anti-Fas. In the absence of inhibitor, this concentration of anti-Fas induced vigorous membrane blebbing within 4 h after stimulation. The cells were exposed to anti-Fas in the presence or absence of Y27632 and examined by phase microscopy. The binding of D56R/S76R and annexin V was analyzed by flow cytometry, as described above.

Results

Binding of scFv to cells in apoptosis

We have recently reported that D56R/S76R, an scFv with specificity for DNA, chromatin, and the phosphatidylserine-$\beta_2$GPI complex, has the ability to bind apoptotic cells (34). To investigate whether D56R/S76R recognizes apoptotic cells following diverse initial stimuli for apoptosis, we examined the binding of D56R/S76R to Jurkat cells treated with staurosporine, camptothecin, or a murine anti-Fas mAb (7C11). These treatments were chosen because they induce apoptosis through different pathways (38–40).

We used flow cytometry for the initial analyses because it provides a broad view of the entire cell population. Following 12 h of incubation in the presence of apoptotic stimuli, apoptosis was examined by staining with annexin V, a molecule that recognizes phosphatidylserine in the presence of Ca$^{2+}$ (Fig. 1). Approximately 29 (camptothecin) to 62% (anti-Fas) of cells treated with annexin V also decreased the staining with annexin V, a molecule that recognizes phosphatidylserine on the surface of apoptotic cells (34). The starting point for our studies was 3H9, a murine autoantibody reactive with DNA, chromatin, and negatively charged phospholipids (35). We observed that binding to the phosphatidylserine-$\beta_2$GPI complex may be enhanced by somatic mutations in the $H$ chain of 3H9 and that additional increases in affinity are achieved by introduction of arginines at sites that sustain such mutations in vivo. In addition, two variants of 3H9, the increased affinity mutant D56R/S76R and the germline revertant R53G/I57T/D65G, demonstrated binding to apoptotic cells.

Positive selection for mutations in 3H9 and other Abs that recognize a complex between phosphatidylserine and $\beta_2$GPI implies direct contacts between B cells and apoptotic cells. To explore the mechanism of Ab binding to apoptotic cells, we used D56R/S76R and compared its binding to DNA4/1, a human anti-DNA autoantibody (36). Confocal microscopy revealed that only D56R/S76R preferentially interacts with blebs on the apoptotic cell surface. Analogous interactions between B cells and apoptotic cells may contribute to the regulation of tolerance to nuclear Ags.
levels. The binding of the scFv to apoptotic cells was mediated by the combining site of D56R/S76R, because the human anti-DNA-derived scFv, DNA4/1 (36), failed to give detectable binding to either annexin V-positive or annexin V-negative Jurkat cells.

Microscopic localization of binding

The finding that the D56R/S76R scFv bound to a subset, but not all, annexin V-positive cells was surprising, because, in vitro, D56R/S76R binds to phosphatidylserine (34), the same anionic phospholipid that mediates the binding of annexin V to apoptotic cells (42). We used immunofluorescence to gain a more detailed view of annexin V and scFv binding to Jurkat cells treated to induce apoptosis. Fig. 2 illustrates the results of confocal microscopy with D56R/S76R or DNA4/1 scFv, annexin V, and TO-PRO-3, a DNA intercalating dye. Each of the three treatments for inducing apoptosis, camptothecin, staurosporine, and anti-Fas, resulted in similar staining morphologies (Fig. 2, A–C). Thus, A–C of Fig. 2 are intended to illustrate representative views seen at different stages of apoptosis, rather than to imply qualitative differences between the treatments.

Cells shown in Fig. 2A were in early to mid-apoptosis, as two of them stained only faintly with annexin V and were larger than the remaining two that stained brightly with annexin V. The D56R/S76R scFv bound preferentially to the cells stained brightly with annexin V. The coincident binding of the two molecules was visualized as areas of yellow, due to the overlap between the Alexafluor 488 (green) signal associated with annexin V and the rhodamine red fluorescence used to localize the scFv. The two annexin V bright cells may represent the two populations of annexin V-positive cells identified by flow cytometry (Fig. 1), as one stained more brightly with annexin V than with the scFv, whereas the other stained equally well with both molecules.

The cells shown in Fig. 2B were in more advanced apoptosis. All three cells were bound by annexin V and the D56R/S76R scFv, although the two molecules did not occupy identical positions on the cell surface but instead were increasingly localized to nonoverlapping membrane domains. This was particularly evident at later stages of apoptosis, as illustrated by the smaller cell near the middle of Fig. 2B whose membrane was permeable to TO-PRO-3.

The segregation of the ligands for annexin V from the epitopes recognized by D56R/S76R was most evident in cells undergoing blebbing. The D56R/S76R scFv bound several large blebs, whereas annexin V occupied areas of the cell membrane that extended between adjacent blebs (Fig. 2C). As a result, areas of overlap between annexin V and the scFv were limited. In addition, a few smaller blebs primarily stained with annexin V. The lower cell shown in Fig. 2C represented an earlier stage of apoptosis in which annexin V staining was already well developed but scFv binding was barely detectable. In cases of limited binding by the scFv, its binding was concentrated to focal points that were near areas of increased annexin V staining.

Fig. 2D illustrates staining with annexin V and reactivity with the human anti-DNA DNA4/1. Of the five cells that stained with annexin V, only two bound the scFv. In each case, scFv binding was observed in the interior of the cells and coincided with the binding of the DNA intercalator TO-PRO-3. This pattern of binding was notably different from the binding of D56R/S76R to the surface of apoptotic cells. The observed differences suggested that the DNA4/1 scFv bound to DNA or chromatin within nuclear fragments rather than to epitopes on the surface of blebs.

Fig. 2, E–G, represent controls for the experiments. Fig. 2E shows that, in the absence of D56R/S76R, annexin V bound efficiently to blebs that formed on the surface of apoptotic cells. Fig. 2F demonstrates that pretreatment with Z-VAD(OMe)-fmk reduced both annexin V and scFv binding to background levels despite treatment with staurosporine, and Fig. 2G shows lack of scFv and annexin V binding to live cells. Although not shown, Z-VAD(OMe)-fmk also greatly reduced binding to cells treated with camptothecin or anti-Fas Ab, thus confirming that D56R/S76R binding requires entry into the caspase-mediated execution phase of apoptosis.
Nuclear fragmentation, blebs, and apoptotic bodies

Studies of human keratinocyte apoptosis have shown that nuclear and cytoplasmic autoantigens segregate into two different classes of cell surface blebs that can be distinguished based on size (5). Binding of D56R/S76R to apoptotic Jurkat cells with intact plasma membranes revealed that the scFv bound preferentially to the surface of both large and small apoptotic blebs (Fig. 3A). To evaluate the location of nuclear fragments relative to the surface blebs bound by the scFv, we took advantage of the observation that, in the later stages of the execution phase of apoptosis, cells lose

FIGURE 2. Confocal immunomicroscopy of cells in different stages of apoptosis. Jurkat cells were treated with camptothecin (A), staurosporine (B), or anti-Fas (C) to induce apoptosis, fixed, and incubated with biotinylated annexin V, the D56R/S76R or DNA4/1 scFv, and the nucleic acid stain TO-PRO-3 (blue). Annexin V was visualized with streptavidin-conjugated to Alexafluor 488 (green) and scFv-protein A fusion proteins were visualized with human IgG conjugated to rhodamine red. All images in this and the following figures (except Fig. 3C) are reconstructed projections of individual optical sections. Areas of overlap between annexin V and the scFv appear yellow, areas of overlap between the scFv and TO-PRO-3 appear magenta, and overlap between all three stains yields white. Flanking A–D, are micrographs that are reduced to one-third the size of the composite image and show the staining with each individual fluorochrome. Not all annexin V-positive cells bind to D56R/S76R (A), and binding of the scFv and annexin V segregate in more advanced stages of apoptosis (B). Cells in the execution phase of apoptosis show binding of D56R/S76R to surface blebs, whereas annexin V is confined to locations between blebs (C). Larger arrowheads in C highlight the most prominent surface blebs bound by D56R/S76R, whereas the smaller arrowhead draws attention to a membrane domain with incipient scFv binding. The human anti-DNA scFv DNA4/1 binds to nuclear fragments in cells that are permeable to TO-PRO-3 (arrowheads), but not to the cell surface (D). In the absence of scFv, annexin V binds to blebs and no staining with rhodamine red is observed (E). Pretreatment of cells with Z-VAD(OMe)-fmk reduces binding of annexin V and D56R/S76R (F). In the absence of proapoptotic stimuli, cells do not stain with annexin V, scFv, or TO-PRO-3 (G).
membrane integrity and become permeable to the DNA intercalator TO-PRO-3 (Fig. 3, B and C). In cells that had become permeable, D56R/S76R bound to blebs that contained nuclear fragments and stained with TO-PRO-3, although we also noted the binding of the scFv to blebs that did not contain nuclear material (Fig. 3, B and C). By analogy to apoptotic keratinocytes, we infer that the smaller blebs that did not stain with TO-PRO-3 (Fig. 3B) contained ribonucleoprotein complexes (5).

Optical sections along the surface of bleeding cells (Fig. 3C) revealed dense packing of DNA in the interior of the larger blebs and the contiguous staining of the bleb surface by the scFv. Strikingly, an analysis of consecutive optical sections indicated that blebs appear to separate from the remainder of the cell. Thus, a link may exist between bleeding and the formation of apoptotic bodies, membrane-bound particles containing nuclear material (Fig. 3C). The proposed link implies that nuclear Ags found in blebs should also be found in apoptotic bodies. This idea is supported by the recent observation that apoptotic bodies retain the capacity to transfer genetic information from apoptotic cells to viable cells (43). The transformation of blebs into apoptotic bodies may be responsible for the reduction in DNA content that is a characteristic of cells in the late stages of apoptosis (44).

**ROCK I kinase facilitates scFv binding to apoptotic cells**

Caspase 3-mediated activation of the Rho-associated kinase ROCK I stimulates the fragmentation of the nucleus and the packaging of nuclear material into blebs at the cell surface. (15, 14). We have exploited the specific inhibitor of ROCK I kinase, Y27632 (45), to evaluate the effects of reduced bleeding on the binding of D56R/S76R to apoptotic cells. For that purpose, cells were treated with anti-Fas in the absence (Fig. 4A) or presence of the inhibitor (Fig. 4B) and examined by microscopy and flow cytometry. In cells that were pretreated with Y-27632, bleeding was inhibited and the binding of D56R/S76R was significantly reduced (Fig. 4C). These results confirmed that blebs play a central role in the binding of D56R/S76R to apoptotic cells. In contrast, pretreatment with Y-27632 did not reduce the staining with annexin V (data not shown). Taken together, our studies indicate that binding of the scFv to blebs requires the same enzyme activity that orchestrates the fragmentation of the nucleus and the migration of nuclear domains to sites of plasma membrane bleeding.

**Discussion**

We examined Ab binding to apoptotic Jurkat cells following treatment with either of three proapoptotic stimuli. We used recombinant Ab fusion proteins: D56R/S76R, derived by in vitro mutagenesis and Ab engineering from 3H9, a murine autoantibody that exhibits dual specificity for DNA and anionic phospholipids (34), and DNA4/1, a human anti-DNA scFv, isolated by phage display from Igs encoded in SLE bone marrow mRNA (36). The binding of D56R/S76R was specific for cells in the execution phase of apoptosis, as inhibition of caspase activity largely blocked the interaction between the scFv and cells (Fig. 1). The most intense binding coincided with blebs that formed on the surface of apoptotic cells, some of which contained lobes of the fragmented nucleus (Figs. 2 and 3). Conversely, binding of the scFv was greatly diminished by inhibition of ROCK I, the kinase that mediates the breakup of the nucleus and the distribution of nuclear fragments to surface blebs (Fig. 4C).

One of the earliest events in apoptosis, the exposure of phosphatidylserine on the outer membrane leaflet, can be visualized by the binding of annexin V (42). Despite the fact that D56R/S76R also binds phosphatidylserine, we found differences in binding to apoptotic cells between the scFv and annexin V. Time course experiments demonstrated that annexin V binding precedes scFv binding, such that the scFv only bound to a fraction of annexin V-positive cells (Fig. 1). The reason for the more selective binding of the scFv was revealed by confocal microscopy. The scFv were more specific than annexin V for cells in the execution phase of apoptosis (Figs. 2 and 3). In addition, at that stage of apoptosis, the two molecules segregated to different binding sites: the scFv bound blebs, whereas, in the presence of the scFv, annexin V predominantly localized to areas between the blebs (Figs. 2 and 3). Because, in the absence of the scFv, annexin V bound to blebs and to

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**FIGURE 3.** Surface blebs that are recognized by the D56R/S76R scFv frequently contain fragments of the nucleus and represent a transition toward apoptotic bodies. Several prominent blebs (arrowheads) are bound preferentially by D56R/S76R (A). D56R/S76R binds to blebs that contain fragments of the nucleus and stain with TO-PRO-3 (large arrowheads in B) as well as to blebs that do not contain nuclear material (small arrowheads in B). Optical section of a blebbing cell shows D56R/S76R binding to the surface of blebs that contain densely packed nuclear material and appear in the process of detaching from the remainder of the cell (arrowheads in C).
areas of the membrane between blebs (Fig. 2E and Ref. 46), the possibility arises that the scFv can displace annexin V from certain domains of the apoptotic cell surface.

Alternatively, the differences in binding between the scFv and annexin V arise because their binding is affected in different ways by additional ligands on the apoptotic cell surface. A ligand that may affect the binding of the scFv to apoptotic cells is β2GPI, a molecule that independently binds to apoptotic cells (27, 47) and enhances the binding of our scFv to phosphatidylserine (34). This alternative predicts that β2GPI also exhibits a preference for binding to blebs on the apoptotic cells surface. We are currently designing experiments to test whether β2GPI serves as a partner in scFv binding to blebs and apoptotic bodies.

FIGURE 4. Pretreatment of cells with the ROCK I kinase inhibitor Y-27632 impairs anti-Fas-induced blebbing and substantially reduces binding of D56R/S76R. Phase microscopy of anti-Fas-treated Jurkat cells reveals actively blebbing cells and the presence of apoptotic bodies (A). Both blebbing and the formation of apoptotic bodies are inhibited by pretreatment with Y27632 (B). The fraction of annexin V-positive cells that also binds D56R/S76R following treatment with anti-Fas (28.5 ± 6.3) is reduced by Y27632 addition (18.3 ± 9.4) (C). Analysis of three independent experiments by paired t tests indicates that the observed reduction is significant (p < 0.05).

Treatment with Y27632, an inhibitor of ROCK-I kinase (45), implied that the preferred ligands for annexin V and D56R/S76R may be different. Following addition of Y27632, anti-Fas-induced membrane blebbing was inhibited and scFv binding was reduced (Fig. 4C), whereas annexin V staining showed no decrease. These results provide evidence that, in apoptosis, the active redistribution of nuclear and cytoplasmic contents into different cellular compartments has a counterpart in the expression of distinct cell surface ligands that can be recognized by autoantibodies. The presence of specific ligands on blebs and apoptotic bodies implies that mechanisms exist to facilitate the recognition and uptake of nucleoprotein complexes that are packaged in blebs and apoptotic bodies during apoptosis.

The differences in binding between scFv and annexin V may be emblematic of two fundamentally different modes of apoptotic cell recognition. One recognition pathway may rely on direct binding to phosphatidylserine to achieve rapid recognition and uptake of apoptotic cells (48). Multiple receptors for phosphatidylserine on phagocytes exemplify this type of interaction (22, 23). Because phosphatidylserine exposure usually (48), but not always (49, 50), precedes blebbing, uptake may already be in progress before cells initiate blebbing. However, the existence of autoantibodies and pattern recognition molecules that specifically bind to blebs suggests that some cells may progress to the execution phase of apoptosis before phagocytosis is complete. Thus, additional recognition pathways may be required to safeguard against the release of immune stimulators from cells in more advanced stages of apoptosis. The additional pathways may use serum proteins as adapter molecules in the recognition of blebs and apoptotic bodies (47, 51).

Binding to blebs is a property that is shared among several pattern recognition molecules that also recognize microbial pathogens, activate complement, and stimulate uptake by phagocytes (20, 52, 53). Molecules that belong to this group and bind to blebs include the mannose-binding lectin (54), the complement collectin C1q (55), C-reactive protein (56), and SAP component (57). These proteins share a rather broad binding specificity that includes carbohydrates, phospholipids, and nucleoprotein complexes, such as chromatin and ribonucleoproteins (52, 55, 57, 58). The binding of pattern recognition molecules to ligands on apoptotic cells may ensure their efficient clearance, as implied by the increased levels of apoptotic cells in mice with C1q (17) or SAP (18) deficiencies.

A situation that is analogous to a deficiency in C1q or SAP may arise if autoantibodies to pattern recognition molecules or their ligands on apoptotic cells disrupt the recognition of apoptotic remnants by cells of the innate immune system (16, 59–62). Impaired removal of apoptotic cell remnants may be a predisposing factor for the production of autoantibodies to nucleoprotein complexes that are typical of lupus (16). As a result of autoantibody binding to apoptotic cells, uptake of cell remnants may be diverted from complement receptors to Fc receptors, thus increasing the likelihood of an autoimmune response. It is also important to consider the possibility B cell receptors, analogous in specificity to D56R/S76R, may engage apoptotic cells. Such interactions may send powerful negative signals and reinforce tolerance to self (63). However, inappropriate activation of B cells capable of binding to apoptotic cells may lead to the presentation of diverse self-Ags that are targeted in SLE. Thus, interactions between B cells and apoptotic cells may represent a critical event in B cell tolerance and autoimmunity.

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