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Apoptotic cell blebs – repositories of autoantigens and contributors to immune context

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Autoimmune rheumatic diseases belong to a diverse group of processes in which multiple tissues are actively injured through the activity of various immune effector pathways. One of the striking observations in these diseases that gives insights into disease mechanism is that, once initiated, the processes amplify over time and are self-sustaining. The amplitude of the disease process typically varies over time, with episodes of more significant disease activity punctuated by periods of inactivity. Such periodic amplification of an immune process (which has characteristic features of an antigen-driven response) suggests that the relevant antigens which drive the disease are periodically exposed. Identifying both the source and pathways underlying such antigen exposure will provide important insights into the mechanisms and causes of rheumatic diseases.

Defining the targets of the immune response in systemic autoimmunity has provided important tools with which to understand the source and mechanisms of antigen exposure. Several principles rapidly became apparent: (i) ubiquitously expressed autoantigens (expressed in all nucleated cells) are targeted, and (ii) specific subsets of autoantigens are preferentially targeted in the different diseases (1, 2). For example, the autoantigens targeted in SLE include nucleosomes and splicing ribonucleoproteins (Sm, RNP). Similarly, components of the translational machinery required for protein synthesis are recognized by antibodies from patients with autoimmune myopathies, and the active centromere is targeted in limited scleroderma. The mechanisms underlying the selection of distinct groups of ubiquitously expressed antigens remain unclear. One novel explanation was proposed about 15 years ago, when it was demonstrated that the autoantigens targeted in SLE were clustered and concentrated in surface blebs of apoptotic cells (3, 4). This observation suggested for the first time that distinct subsets of autoantigens targeted in different disease phenotypes might share properties during a specific physiologic process (e.g. programmed cell death) (5). The studies also suggested that the targeted antigens would share properties that rendered them more likely to initiate an immune response (e.g. changes in structure or adjuvant properties) (6). Numerous findings supporting and extending this hypothesis have been generated over the past decade. This data can now be assembled into a framework within which to understand and investigate the initiation and propagation of systemic autoimmunity.

Apoptosis is a form of programmed cell death where various signals from inside and outside the cell are integrated prior to initiation of an amplifying biochemical program that leads to a stereotyped series of biochemical and morphological changes. Under most circumstances, apoptotic cells are rapidly recognized and innocuously cleared by surrounding cells. An important determinant of such recognition and clearance are changes of the apoptotic cell surface, which undergoes early redistribution of phospholipids and other components, resulting in different binding properties for soluble and cell-associated partners. Soluble factors include early classical pathway complement components and CRP, which appear to play a role in rendering early apoptotic cells anti-inflammatory and immunosuppressive (7). There is now significant evidence that apoptotic cells generated during normal cell turnover are efficiently cleared by macrophages and dendritic cells, and that such clearance is

associated with active suppression of the immune response to antigens within these cells (8). It is very striking that delayed or abnormal clearance of apoptotic cells can result in dendritic cell (DC) maturation, and is associated with systemic autoimmunity in both humans and animals (9, 10). Although there is preferential clustering of lupus autoantigens within surface blebs of apoptotic cells, understanding whether these blebs have distinct immune properties remains unknown.

In this issue of Arthritis and Rheumatism, Berden and colleagues provide additional insights into whether different populations of apoptotic remnants (i.e. apoptotic blebs consisting of clustered and concentrated autoantigens compared to apoptotic corpses) have distinct immune effects. Of note, there is some confusion in the literature about the term apoptotic body. This term has been used to describe both the larger, organelle-containing membrane-bound structures that come off apoptotic cells, as well as the residual corpse remaining after surface blebs have separated. To clarify this, we refer to the latter group as “apoptotic corpses”, and the former as “apoptotic bodies”. Using a murine model, the investigators generate apoptotic cells by incubating them *in vitro* with 4-nitrouinolone 1-oxide for 24 hours prior to separation of blebs from the remaining “corpses” by differential centrifugation. They then compared the effects of these different apoptotic populations on maturation of myeloid DCs and T cell stimulatory capacity. They found that apoptotic blebs, but not the residual apoptotic corpses, were efficiently ingested by DCs, induced subsequent upregulation of costimulatory molecule expression, and activated T cells in an allo-MLR. These effects were chloroquine-insensitive, suggesting that endosomal TLR signaling was not required.

These studies therefore suggest that autoantigen-rich apoptotic blebs which are not efficiently cleared might have two important properties which determine their subsequent immune fate: (i) high concentration of autoantigens and (ii) adjuvant effect on DCs. Exactly when the latter property is acquired is not clear, nor are the actual mechanisms and pathways underlying such an adjuvant effect known. Accumulating data in other systems demonstrates that intracellular autoantigens released from cells may under some circumstances express chemoattractant and adjuvant properties. For example, histidyl-tRNA synthetase ligates CCRxx and is chemoattractant to immature dendritic cells and monocytes (11). Similarly, the DNA- and RNA-containing autoantigens targeted in SLE may ligate various Toll-like receptors (TLRs), and regulate the immune response (12). The insensitivity of the adjuvant effects of apoptotic blebs to chloroquine in this study strongly suggests that TLR ligation is not playing a prominent role here. It is possible that various autoantigen modifications during various physiologic states and different forms of cell death are responsible for DC activation. Defining the relevant ligands and receptors that mediate this effect may provide important therapeutic opportunities.

Extrapolating observations made with dying cells generated *in vitro* to phenomena occurring naturally *in vivo* is very challenging, and such studies are potentially subject to numerous criticisms. These include (i) the physiologic relevance of the stimulus used to induce cell death, (ii) how reproducible and representative the sub-apoptotic particles used are of structures generated *in vivo*, (iii) assumptions about the circumstances which would generate such material *in vivo*; (iv) the validity of the aging and purification approaches, and (v) the homogeneity of the preparations. In spite of such criticisms, initial definition of key interactors and pathways *in vitro* may identify experiments and approaches to confirm that these pathways are relevant to disease pathogenesis *in vivo*. For example, it will be important to show that DCs are mature and activated at sites with large amounts of uncleared apoptotic material *in vivo*. If such upregulation of costimulatory molecules on DCs is important in initiating and propagating immune responses to apoptotic blebs in various models of systemic autoimmunity, inhibitors of costimulation might have

therapeutic utility. Similarly, it will be important to demonstrate– in an antigen-specific way with endogenous or model antigens – that aged apoptotic blebs *in vivo* can efficiently initiate T cell activation, autoantibody responses, and potentially autoimmune tissue dysfunction. This will provide support for the importance of this mechanism in autoimmune phenotypes.

One of the interesting and unexpected findings in the study by Berden et al is that Th17 T cells are induced by bleb-activated DCs. IL-17 producing helper T cells are a relatively new class of inflammatory T cells which have been increasingly implicated in inflammatory disease pathogenesis, including arthritis, multiple sclerosis and SLE. While Th17 cells are generated in the allo-MLR in these studies, mice and humans differ significantly in the signals required for Th17 differentiation and expansion, making it important to confirm these findings in humans prior to extrapolating to the human system.

These studies support the idea that the proinflammatory properties of apoptotic cells are limited to the apoptotic structures in which autoantigens are clustered, and that it is these components which can affect dendritic cell biology. The effect of different inducers of apoptosis on the exact morphology, sequence, and pro-inflammatory effects of apoptotic death may be important factors that define the immune fate of apoptotic cells. Unfortunately, these effects remain largely unknown. Previous studies have suggested that the downstream consequences of different death inducers are nuanced. Wiegand et al showed that the apoptotic stimulus (etoposide vs. staurosporine) played a significant role in how effectively phagocytes could recognize and phagocytose apoptotic cells (13). Different stimuli may thus affect the kinetics of death and degree of blebbing, and determine whether cells are cleared without inciting an immune response. It will be important to define how different stimuli affect the kinetics of blebbing and subsequent cell death. Stimuli that induce a high degree of blebbing may result in the inability of phagocytes to efficiently sequester autoantigen-containing debris from activating immune responses. It is also important to underscore the relevance of the cell which is dying, about which little is currently known.

The study by Berden and colleagues does not address whether blebs from early apoptotic cells have the same immune effects as those from aged apoptotic cells. Available data would suggest that early apoptotic cells do not activate DCs, suggesting that time-dependent changes likely play some role in the acquisition of adjuvant properties. Clearly, the ability of such time-dependent changes to manifest themselves in vivo is related to efficiency of clearance. Whether first order or zero order kinetics apply at various rates of flux through the apoptotic clearance pathway remains unknown, but is very important to determine. A good comparison would be the patient treated with therapeutic doses of aspirin – initially metabolism is first order (when more drug is given, there is a proportionate increase in clearance, and serum drug levels increase linearly); once pathways are saturated, a very small increase in aspirin dose results in large increases in serum levels. It is not currently known whether apoptotic cell clearance is first order at all rates of flux *in* vivo, whether and how this might be regulated, and whether there are differences in different tissues and microenvironments. These factors are clearly relevant to the initiation and amplification of autoimmunity. For example, if, at low rates of flux, there is more than enough capacity to clear all apoptotic cells, the chances of initiating autoimmunity may be low. If there is a saturable component *in vivo*, however, a relatively small increase in the flux through the pathway may result in a substantial accumulation of uncleared cells, providing an opportunity for these cells to be subjected to additional time-dependent modifications in antigen structure and adjuvant properties. It is particularly interesting that impaired apoptotic cell clearance can render animals susceptible to systemic autoimmunity. Tingible body macrophages require MFG-E8 for efficient uptake and clearance of apoptotic cells, a process which is markedly deficient in mice lacking milk factor globule EGF-like 8 (MFG-

E8), where a large number of uncleared apoptotic cells are found in lymph nodes, and mice manifest a lupus-like illness with high titer ANA and renal disease (14). Similarly, C1q-deficiency appears to be accompanied by decreased clearance of apoptotic cells and systemic autoimmunity in both mice and humans (9).

In summary, accumulating data implicates dying cells as repositories of the autoantigens and immune context that drive systemic autoimmunity. The data by Berden et al show that blebs (rather than residual bodies) from aged apoptotic cells activate mouse DCs, and induce T cell activation, including Th17 cells. Understanding whether, where and how such structures are generated *in vivo*, and the mechanisms underlying their adjuvant properties, are major priorities.

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