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REVIEW

Disturbances of apoptotic cell clearance in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus is a multifactorial autoimmune disease with an as yet unknown etiopathogenesis. It is widely thought that self-immunization in systemic lupus is driven by defective clearance of dead and dying cells. In lupus patients, large numbers of apoptotic cells accumulate in various tissues including germinal centers. In the present review, we discuss the danger signals released by apoptotic cells, their triggering of inflammatory responses, and the breakdown of B-cell tolerance. We also review the pathogenic role of apoptotic cell clearance in systemic lupus erythematosus.

Introduction

Cell death is a necessary and natural process that occurs under both physiological and pathological conditions in all tissues. This process is also a critical mechanism in immune tolerance and in contraction of normal immune responses to exogenous antigens. Apoptosis is the predominant pathway for cell death, but cells can also undergo death through necrosis and necroptosis, a programmed cell death independent of caspase activation [1]. Apoptotic cells must be quickly removed from tissues so that they elicit neither inflammation nor immune responses. Inefficient clearance of apoptotic cells and subsequent accumulation of apoptotic cell debris provoke a chronic inflammatory response and may lead to breakdown of self-tolerance [2].

Autoimmunity in systemic lupus erythematosus (SLE) is believed to be driven by autoantigens. Certain key autoantigens are demonstrable on blebs of apoptotic cells. Considerable evidence supports the notion that SLE autoimmunity could be related to impaired or delayed clearance of apoptotic cells. Persistent apoptotic cells

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may progress to secondary necrotic cell death, a process associated with danger signals, accessible modified autoantigens, and initiation of autoimmune reactions.

Clearance of apoptotic cells

Efficient recognition and clearance of apoptotic cells involve many ligands, receptors, and bridging molecules between dying cells and phagocytes. *In vitro* studies have indicated that this process is tightly controlled and remarkably complex.

Early apoptotic cells express eat-me signals through asymmetric distribution of aminophospholipids such as phosphatidylserine (PS), phosphatidylethanolamine, and phosphatidylcholine to the outer leaflet. PS is the best-characterized eat-me signal, and externalization of PS alone is efficient enough to initiate the engulfment of apoptotic cells by macrophages (Mφs) [3]. In addition, apoptotic cells actively secrete chemoattractant factors known as find-me signals to the environment to attract phagocytes, such as Mφs and dendritic cells (DCs). The covalent dimer of ribosomal protein S19 was the first identified find-me signal of apoptotic cells [4], but its mechanism of action is still unclear. Apoptotic thymocytes release nucleotides (ATP and UTP) in a caspase-dependent manner to attract monocytes [5], so that release of nucleotides can be blocked by the caspase inhibitor zVAD-fmk. A diverse find-me signal has been identified and is reviewed elsewhere [6]. Interestingly, a recent study reported that the 75 to 80 kDa iron-binding protein lactoferrin could serve as an anti-attraction (keep-out) signal by different apoptotic cell lines. Lactoferrin mediates strong inhibition of neutrophil migration but not Mφ migration [7]. SIRP-α, another keep-out signal, was reported by Tsai and Discher [8]. CD47-SIRP-α signaling negatively regulates phagocytosis through inhibition of the downstream signaling events of phagocytic receptors. Loss of function or blocking of either molecule causes removal of viable cells that are not normally phagocytosed [9].

Recognition and binding of find-me signals on the apoptotic cells initiate the engulfment of apoptotic cells. Receptors such as CD36, scavenger receptor A, and PS receptor can directly bind to the eat-me molecules and

initiate phagocytosis, while other receptors require the help of bridging molecules (reviewed in [10,11]). Tyro-3, Axl, and Mertk (TAM) receptor tyrosine kinases bind to apoptotic cells with the aid of two vitamin K-dependent factors, growth arrest specific protein 6 and protein S [12,13]. Mice lacking Mertk have impaired clearance of apoptotic cells and develop lupus-like autoimmunity, while TAM triple-knockout mice suffer from more severe disease [14,15]. These mice develop a systemic auto-immune disorder with swollen joints and footpads, skin lesions, and glomerular immune complex deposition. A broad spectrum of autoantibodies is detectable in the serum. TAM receptor signaling normally not only facilitates apoptotic clearance, but also downregulates proinflammatory Mφs and DCs. Activation of TAM receptor signals leads to a broad inhibition of Toll-like receptor (TLR)-induced cytokines through the IFNAR-STAT1 cassette to induce TLR suppressors SOCS1 and SOCS3 [16].

Defective clearance of apoptotic cells can result in accumulation of apoptotic debris. In the thymus and bone marrow, most developing T cells and B cells under-go apoptosis due to failure of positive and negative selec-tion. Those apoptotic cells have to be cleared efficiently in a non-inflammatory way. Tisch and colleagues demon-strated a key role of Mertk on T-cell central tolerance by enhancing negative selection of autoreactive T cells specific for pancreatic β cells [17]. Nonobese diabetic mice lacking Mertk failed to develop diabetes due to an increased thymic negative selection of β-cell-specific T cells and an increased capacity of DCs to induce thymocyte apoptosis [17].

In germinal centers, clonal selection of high-affinity B cells results in large numbers of apoptotic cells, includ-ing autoreactive cells. Clearance of these cells is essential to maintain peripheral tolerance. Specialized phagocytes that clear dying cells are referred to as tingible body macrophages (TBMφs) in the germinal center. Nagata's group revealed a critical role of milk fat globule epidermal growth factor 8 (MFG-E8) in integrin-mediated TBMφ phagocytosis of apoptotic cells [18]. MFG-E8 promotes the phagocytosis of apoptotic cells by serving as a bridging molecule between apoptotic cells and phago-cytes. Without MFG-E8, mice developed autoimmune disease associated with defective apoptotic cell clear-ance in the germinal center. Alternatively, when apop-totic cell clearance was disturbed with MFG-E8 mutant protein D89E, autoantibodies against phospholipids and nuclear proteins were induced in mice [19]. Later, the same group reported a reduced level of MFG-E8 in some childhood-onset and adult SLE patients and a dose-dependent inhibition of Mφ uptake of apoptotic cells by human MFG-E8 [20]. Aberrant splicing of MFG-E8 was also reported in SLE patients. Repeated administration of

the same mutant MFG-E8 into mice induced anti-cardiolipin and antinuclear autoantibodies [21]. Similarly, we discovered a primary role of Mertk on TBMφ clearance of apoptotic B cells in the germinal center. Large numbers of apoptotic bodies accumulated outside TBMφs in Mertk$^{-/-}$ mice immunized with NP-chicken γ-globulin [22].

The T-cell immunoglobulin mucin (TIM) gene family has recently been shown crucial for clearance of apoptotic cells, autoimmune regulation, and tolerance induction. Tim-1 and Tim-4 specifically bound PS on the surface of apoptotic cells [23]. Tim-4$^{-/-}$ mice show defects in apoptotic cell clearance by peritoneal Mφs and B-1 cells. Tim-4$^{-/-}$ mice develop autoantibodies against dsDNA associated with hyperreactive T cells and B cells [24]. Tim-3, on the other hand, recognizes apoptotic cells via the FG loop in the IgV domain. Inhibition of apoptotic cell engulfment by CD8$^+$ DCs through anti-Tim-3 mAb results in reduced autoantigen cross-presentation *in vivo* and *in vitro*. Administration of Tim-3 and Tim-4 mAb induces autoantibody production in animals [25].
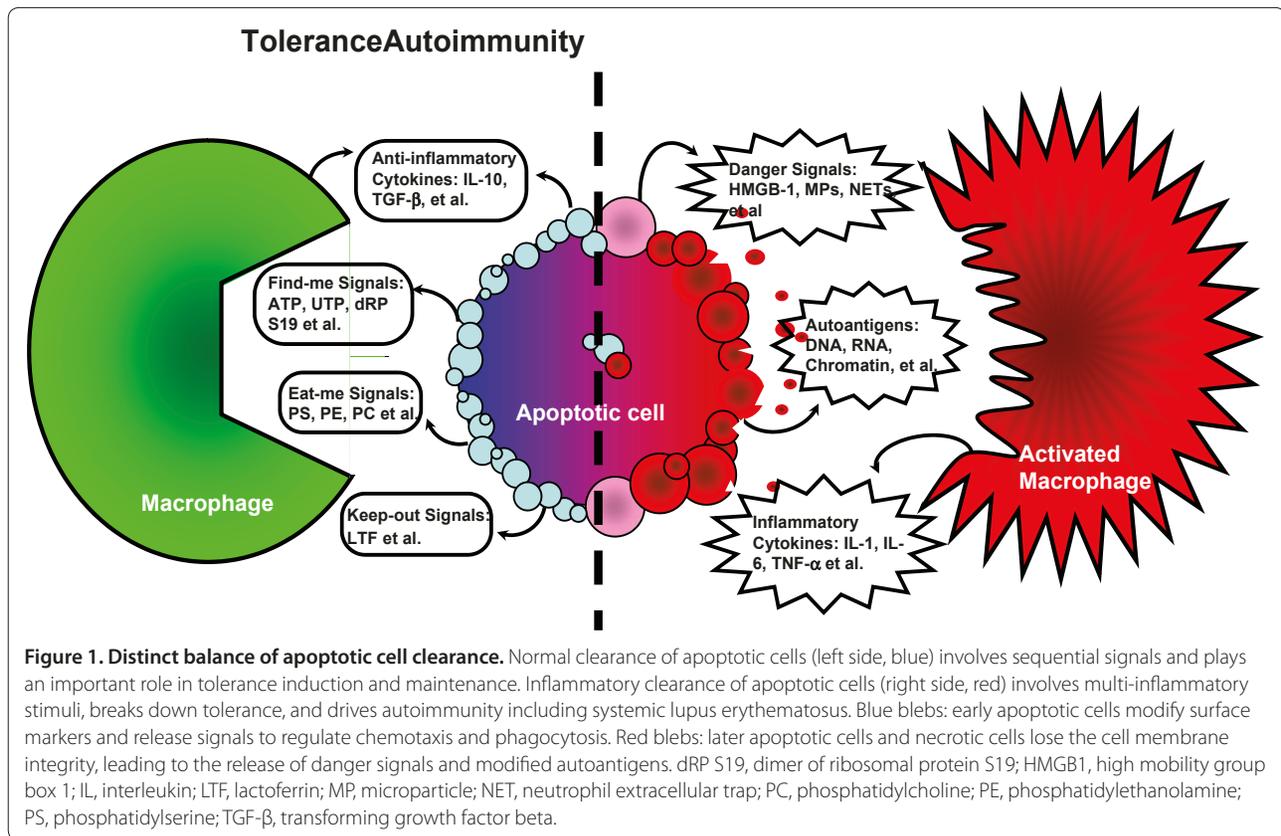
Danger signals from later apoptotic cells/necrotic cells

Phagocytic clearance of apoptotic cells can influence immune responses dramatically by enhancing or suppres-sing inflammation, depending on additional immune stimuli (Figure 1). DCs may present self-derived antigen if appropriate danger signals are provided. Defective or prolonged clearance of apoptotic cells allows them to progress to secondary necrosis, a stage where danger signals may accumulate. Our knowledge about danger signals released from apoptotic cells, however, is rather limited. Nucleic acid, proteins, inflammatory cytokines, and metabolic intermediates are all well-accepted danger signals in other contexts [26]. We highlight recently described key danger signals in the present review.

High mobility group box 1 protein

High mobility group box 1 (HMGB1) is an evolutionary conserved ubiquitously expressed chromosomal protein consisting of two positively charged DNA binding domains, called HMG boxes A and B.

HMGB1 binds to and helps to stabilize dsDNA, ssDNA, distorted DNA, and nucleosomes. HMGB1 can be actively secreted from living cells and passively secreted from dying cells during late apoptosis and necrosis (reviewed in [27]). HMGB1 released from apop-totic cells undergoes post-translational oxidative modifi-cation and acts as a proinflammatory mediator [28]. HMGB1 has been found to be elevated significantly in sera from lupus patients [28]. The precise patho-physiological role of HMGB1 in lupus, however, remains to be further elucidated. In UV-induced skin lesions of

Figure 1. Distinct balance of apoptotic cell clearance. Normal clearance of apoptotic cells (left side, blue) involves sequential signals and plays an important role in tolerance induction and maintenance. Inflammatory clearance of apoptotic cells (right side, red) involves multi-inflammatory stimuli, breaks down tolerance, and drives autoimmunity including systemic lupus erythematosus. Blue blebs: early apoptotic cells modify surface markers and release signals to regulate chemotaxis and phagocytosis. Red blebs: later apoptotic cells and necrotic cells lose the cell membrane integrity, leading to the release of danger signals and modified autoantigens. dRP S19, dimer of ribosomal protein S19; HMGB1, high mobility group box 1; IL, interleukin; LTF, lactoferrin; MP, microparticle; NET, neutrophil extracellular trap; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TGF-β, transforming growth factor beta.

experimental cutaneous lupus, HMGB1 increased coincident with clinical activity [29,30]. HMGB1 can interfere with apoptotic clearance by blocking cell surface PS, causing accumulation of apoptotic cells and worsening disease. HMGB1 can also increase production of IFNα by plasmacytoid DCs through TLR9 and RAGE, or of cytokines from activated B cells through TLR2. HMGB1 has been identified as an autoantigen in lupus patients, and thus may itself be a target in autoimmune responses. Anti-HMGB1 autoantibodies are positively correlated with the SLE disease activity index [31]. Patients shown to have positive autoantibodies for HMGB1 presented with significantly higher SLE disease activity index values.

Microparticles

Microparticles (MPs), also known as plasma membrane-derived vesicles, are small membrane-bound vesicles released from dying cells via blebbing and shedding. MPs incorporate cytoplasmic proteins such as cytokines, growth factors, acute phase proteins, and DNA and RNA.

Increased numbers of plasma MPs have been documented in SLE patients with secondary anti-phospholipid syndrome [32,33]. Pisetsky and Lipsky proposed an autoadjuvant model for MPs as a revised version of the waste disposal hypothesis of SLE [34]. In this model,

nucleic-acid-containing MPs act as autoadjuvants in both central tolerance and peripheral B-cell activation in normal individuals. MPs loaded with DNA and RNA enhance their stimulatory capacity and ensure effective central deletion during B-cell development. In individuals with SLE, MP may particularly promote the survival of DNA-specific and RNA-specific autoreactive B cells due to their effectiveness in stimulating B cells through their immunoglobulin receptors. Recently, Antwi-Baffour and colleagues revealed a dose-dependent competition of MPs with apoptotic cells for PS receptor on Mφs [35]. The fact that MPs express high concentrations of PS on their outer leaflet makes them potent inhibitors of phagocytosis of normal apoptotic cells. This may result in secondary necrosis and subsequent increase of apoptotic debris.

To our knowledge, there is no universally accepted definition for MPs. A wide range of MP sizes (0.02 to 2 μm) has been reported by different groups in the literature [34,36]. MPs bear many surface adhesion molecules, making them likely to adhere to leukocytes or endothelial cells. This and other physical characteristics of MPs add to difficulties in their quantitative analysis, emphasizing the need for attention to collecting, processing, and storage of samples containing MPs. Flow cytometry-based assays are routinely used in the field, while electron or confocal microscopy may be better for

visualization, characterization, and morphological study [36]. An ELISA-based assay was also developed for platelet-derived MPs by Osumi and colleagues [37].

Neutrophil extracellular traps

Neutrophil extracellular traps (NETs), extended chromatin networks released from activated neutrophils, constitute an intricate mechanism used by neutrophils to capture and kill invading microorganisms [38]. In SLE patients, neutrophils are activated and express genes induced by type I interferons [39,40]. SLE patients produce antibodies against NETs and against neutrophil proteins such as myeloperoxidase and proteinase-3 [41]. Impaired recognition and clearance of apoptotic neutrophils may due to reduced expression of CD44 on neutrophils in SLE [42]. NETs are made of DNA, histones, and neutrophil proteins. Timely removal of NETs is essential to avoid self-antigen presentation. Zychlinsky and colleagues found that impaired degradation of NETs in SLE patients correlates with lupus nephritis. Difficulty in NET clearance is due to the presence of DNase 1 inhibitors and prevention of DNase 1 access to NETs by anti-NET antibodies [39].

DNase I

DNase I facilitates chromatin breakdown during apoptosis and has been implicated in the pathophysiology of SLE since the 1950s. Lupus-prone NZB/NZW mice have significantly lower serum and urine concentrations of DNase I compared with normal mice [43], which may allow more immune complexes to persist in the kidney and further promote disease progression. An elegant study with the same model showed that reduced levels of renal DNase I coincided with deficient fragmentation of chromatin from dead cells, implying that the lack of this enzyme may have caused delayed clearance [44]. Furthermore, DNase-I-deficient mice developed a SLE-like syndrome with antinuclear antibodies, immune complex deposition, and glomerulonephritis [45]. Consistent with these findings, lower serum DNase I activity was demonstrated in SLE patients compared with patients with rheumatoid arthritis and scleroderma and compared with healthy controls [45,46]. In this regard, two DNase-I-deficient SLE patients with high serum titers of anti-dsDNA antibodies were reported [47]. Attempts to use DNase I as a therapeutic agent in SLE were carried out by several groups and were mildly encouraging [48,49].

Disturbed apoptotic cell clearance in SLE patients

Much of the preceding evidence for apoptotic clearance impairment in SLE comes from mouse models of lupus. In human lupus, apoptotic cell bodies unengulfed by lymph node TBMφs have been observed *in vivo*. Free apoptotic debris found attached to follicular DCs might provide survival signals to autoreactive B cells generated from random somatic hypermutation [50]. Significantly increased numbers of circulating endothelial cells in SLE patients have been interpreted to reflect an enhanced apoptotic rate, and possibly decreased clearance of these cells [51]. Freshly isolated or cultured lymphocytes from SLE patients showed a significantly increased fraction of cells undergoing apoptosis in comparison with normal control individuals or rheumatoid arthritis patients, with a correlation between SLE disease activity and the rate of *in vitro* apoptosis. Extracellular nucleosomes were also increased in proportion to the rate of apoptosis [52]. In juvenile-onset SLE, neutrophil apoptosis is significantly increased, and this is thought to be due to an imbalance in proapoptotic and antiapoptotic factors in both neutrophils and sera from the patients [53].

UVB has long been associated with apoptosis induction and lupus flares. Caricchio and colleagues revealed a dose-dependent immunological fate of UVB-induced apoptosis: non-inflammatory apoptosis from low-dose UVB, and proinflammatory apoptosis from higher-dose UVB [54]. Later, Reefman and colleagues compared the apoptotic/necrotic rate of 14 SLE patients with 16 controls in response to 200 MJ/cm^2 UVB irradiation. Although sensitivity of SLE to UVB was not related to the numbers of apoptotic or necrotic keratinocytes in the skin [55], UVB-induced skin lesions were associated with apoptotic keratinocytes [56]. In another study, Fas-mediated apoptosis was thought to account for the low numbers of bone marrow CD43$^+$ cells in active SLE, although relatively small numbers of patients were studied [57].

An *in vitro* defect in apoptotic cell clearance has been described in SLE. Mφs cultured from SLE patients display morphological abnormalities with impaired phagocytosis of apoptotic cells [50,58]. Mφs derived from SLE monocytes or stem cells were smaller with less ability to differentiate and with impaired adhesion [58]. Moreover, sera from SLE patients possessed an enhanced capacity to induce apoptosis [59]. In this study, sera from 37 sex-matched and age-matched SLE patients and a total of 37 other autoimmune/infectious disease and healthy donors were sampled; the apoptosis-inducing effect of the sera on normal monocytes or lymphocytes was compared. The apoptosis-inducing effect was significantly higher in sera from SLE patients than controls. Subsequent studies showed that the SLE sera-induced apoptosis is caspase dependent but death receptor independent [60]. Although the apoptosis-inducing factor has not yet been identified, the existence of these factors may give rise to apoptotic cells in SLE and worsen the pathogenic condition. Interestingly, Dransfield and colleagues found that glucocorticoid-treated Mφs have increased capacity to phagocyte apoptotic neutrophils, apparently due to enhanced protein S signaling of the

Mertk [61]. In this regard, we recently reported low levels of protein S in certain subsets of SLE patients [62]. Together, the data may support an important role of Mertk in the inefficient clearance of apoptotic cells in certain SLE patients. A follow-up investigation of levels of TAM receptors in SLE patients may help in understanding the distinct role of TAM receptors in the pathogenesis of SLE.

There are strong links between apoptotic clearance deficiency and SLE, yet no direct etiological role has been established for impairment of apoptotic clearance in human SLE. The initiating phase of SLE may be multifactorial and individually variable, while the presence of excessive apoptotic debris may have profound pathogenic relevance and correlation with disease activity. It has been shown that the presence of antinuclear antibodies, reactive with apoptotic debris, may render apoptotic cells proinflammatory and thus create a vicious cycle producing further autoantibodies and inflammation [63]. In this regard, the lupus erythematosus cell exemplifies this phenomenon of phagocytosis of autoantibody-coated apoptotic cells. Lupus erythematosus cells were discovered over 60 years ago, and the LE prep, where lupus white blood cells are observed to undergo spontaneous phagocytosis of debris *in vitro*, was the standard test for lupus until about 30 years ago [64].

Conclusions

Clearance of apoptotic cells is a daunting challenge to the immune system, which has multiple redundant receptor–ligand systems to efficiently eliminate senescent and superfluous apoptotic cells. It is now well established in animal models that this process may fall short under certain circumstances, leading to abnormally persistent and potentially immunogenic apoptotic and necrotic cells. Both *in vivo* and *in vitro* evidence supports the notion that certain human autoimmune diseases, particularly SLE, may also arise from impaired apoptotic cell clearance. Better understanding of the immunobiology of apoptotic cell clearance may lead to new therapeutic approaches to lupus and related autoimmune diseases.

Autoimmune Basis of Rheumatic Diseases

This article is part of a series on *Systemic lupus erythematosus*, edited by David Pisetsky, which can be found online at http://arthritis-research.com/series/lupus

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Abbreviations

DC, dendritic cell; dsDNA, double-strand DNA; ELISA, enzyme-linked immunosorbent assay; HMGB1, high mobility box 1; IFN, interferon; Mφ, macrophage; mAb, monoclonal antibody; Mertk, Mer receptor tyrosine kinase; MFG-E8, milk fat globule epidermal growth factor 8; MP, microparticle; NET, neutrophil extracellular trap; PS, phosphatidylserine; SLE, systemic lupus erythematosus; ssDNA, single-strand DNA; TAM, Tyro-3, Axl, and Mertk; TBMφ, tingible body macrophage; TIM, T-cell immunoglobulin mucin; TLR, Toll-like receptor; TNF, tumor necrosis factor; UVB, ultraviolet B.

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