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Efferocytosis and autoimmune disease

Mahiru Kawano[1,2] and **Shigekazu Nagata**[1]

[1]Laboratory of Biochemistry & Immunology, World Premier International Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan
[2]Present address: Department of Obstetrics and Gynecology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

Correspondence to: S. Nagata; E-mail: snagata@ifrec.osaka-u.ac.jp

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REVIEW

Abstract

An enormous number of cells in the body die by apoptosis during development and under homeostasis. Apoptotic cells are swiftly engulfed by macrophages and digested into units. This removal of apoptotic cells is called 'efferocytosis'. For efferocytosis, macrophages recognize phosphatidylserine (PtdSer) exposed on the cell surface as an 'eat me' signal. In healthy cells, PtdSer is exclusively localized to the inner leaflet of the plasma membrane by the action of flippases. When cells undergo apoptosis, caspase cleaves flippases to inactivate them, while it cleaves pro-scramblases to active scramblases, which quickly translocate PtdSer to the cell surface. The PtdSer is then recognized by PtdSer-binding proteins or by PtdSer receptors on macrophages, which subsequently engulf the apoptotic cells. When efferocytosis fails, apoptotic cells can rupture, releasing cellular materials that can evoke an autoimmune response. Thus, a defect in the PtdSer-exposing or PtdSer-recognizing processes triggers autoimmunity, leading to a systemic lupus erythematosus-type autoimmune disease.

Keywords: apoptosis, macrophages, phosphatidylserine, scramblase, SLE

Introduction: phosphatidylserine as an 'eat me' signal for efferocytosis

Many excess cells are generated and die during animal development (1). Most cells in the body have a finite lifespan, and billions of senescent cells die every day to maintain homeostasis (2). This cell death occurs through apoptosis, in which a cascade of cysteine proteases called caspases is activated, eventually leading to the cleavage of >500 cellular substrates (3). Although a large number of cells undergo apoptosis, it is not easy to detect the dying cells *in situ*, because apoptotic cells are swiftly cleared by a process called 'efferocytosis', in which they are engulfed by macrophages (4). Macrophages do not engulf living cells, indicating that the apoptotic cells must express an 'eat me' signal(s) (5). Among numerous molecules proposed to be the 'eat me' signal, the strongest candidate is phosphatidylserine (PtdSer) (2, 6–8). Here we describe how PtdSer is exposed on the surface of apoptotic cells, and how it is recognized by macrophages for engulfment of dead cells. We then discuss the possibility that a failure in this process activates autoimmunity.

Phospholipid flippases and scramblases

PtdSer is a glycerophospholipid. All glycerophospholipids have two fatty acids and a phosphoric acid attached to a glycerol via an ester bond. A serine, choline or ethanolamine is joined to the phosphate to produce PtdSer, phosphatidylcholine (PtdCon) or phosphatidylethanolamine (PtdEtn), respectively. Phospholipids have amphiphilic characteristics (a hydrophobic tail consisting of fatty acids, and a hydrophilic head region containing phosphoric acid) and form the bilayer structure of the plasma membrane. Interestingly, these glycerophospholipids are asymmetrically distributed between the inner and outer leaflets of the plasma membrane, probably to maintain the membrane integrity and/or for signal transduction (9). PtdSer and PtdEtn are confined to the inner leaflet, whereas PtdCon is distributed at a ratio of 6:4 between the outer and inner leaflets. This asymmetrical distribution of PtdSer and PtdEtn is maintained by flippases that translocate them from the outer to inner leaflet in an ATP-dependent manner (9, 10). We recently reported that two ubiquitously expressed members of the P4-type ATPase family (ATP11A and ATP11C) serve as flippases at the plasma membrane (11, 12).

The middle of the ATP11A or ATP11C molecule contains two or three recognition sites for caspase 3, the most downstream caspase in the cascade, and these flippases are cleaved during apoptosis (11, 12) (Fig. 1). This cleavage inactivates the flippase activity, but it is not sufficient to expose PtdSer. This is because once the asymmetrical distribution of phospholipids is established, it is difficult and takes days to spontaneously break

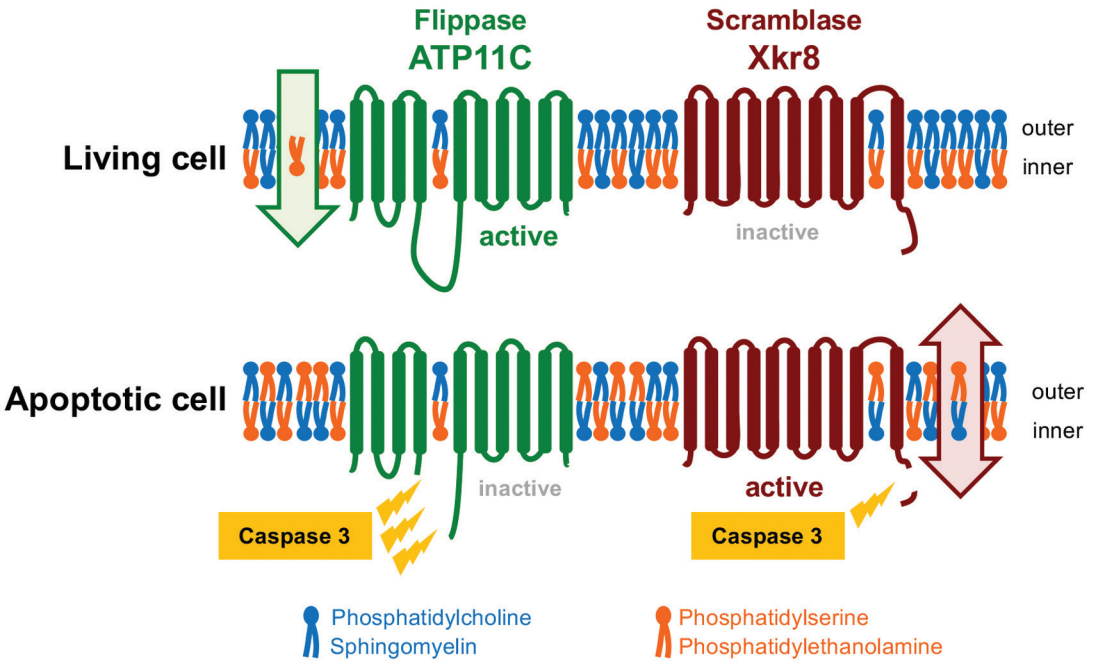

Fig. 1. Flippase- and scramblase-mediated regulation of apoptotic PtdSer exposure. ATP11A and ATP11C are ubiquitously expressed P4-type ATPases that contain 10 transmembrane regions. In living cells, these proteins actively translocate PtdSer from the outer to the inner leaflets, maintaining the asymmetrical distribution of PtdSer. When cells undergo apoptosis, caspase 3 cleaves ATP11A and ATP11C, and irreversibly inactivates them. Simultaneously, caspase 3 cleaves Xkr8 and activates it. Active Xkr8 seems to provide a path for phospholipids to translocate the hydrophobic layers, causing the rapid and irreversible PtdSer exposure in apoptotic cells. Blue, PtdCon and sphingomyelin; red, PtdSer and PtdEtn.

it, because the polar head group has to travel through the hydrophobic lipid layer to reach the outer leaflet (13). Therefore, to quickly expose PtdSer on the cell surface, phospholipid scramblases bidirectionally and non-specifically translocate phospholipids between the two leaflets of the plasma membrane (9).

There are two types of scramblases: Ca^{2+}-dependent and caspase-dependent (14, 15). The scramblase responsible for the apoptotic PtdSer exposure is a caspase-dependent scramblase of the XK-related (XKR) family. Three members of the Xkr protein family (Xkr4, Xkr8 and Xkr9) have been identified as caspase-dependent phospholipid scramblases (14, 16). These are membrane proteins carrying 10 transmembrane segments, and contain a caspase-recognition site at their C-terminus. Whereas Xkr8 is ubiquitously expressed, Xkr4 and Xkr9 are expressed tissue-specifically, mainly in the nervous system and intestines, respectively. Xkr8 forms a complex with basigin (BSG) or neuroplastin (NPTN), which functions as a chaperone to translocate Xkr8 from the endoplasmic reticulum to the plasma membrane (17). To gain scramblase activity, Xkr8 is cleaved by caspase 3 (Fig. 1) and the Xkr8–BSG or Xkr8–NPTN complex then forms a higher-order complex that is active.

PtdSer exposed by the activated scramblase alone does not function as an 'eat me' signal (18). Either the exposed PtdSer under these conditions returns quickly to the inner leaflet because of flippase activity or it has a reduced ability to bind the PtdSer receptor on macrophages. However, as mentioned above, the ATP11A and ATP11C flippases are inactivated by caspase during apoptosis. In this case, once PtdSer is exposed to the cell surface by the Xkr8 scramblase, it cannot be returned to the inner leaflet, and serves as the 'eat me' signal. PtdSer is exposed not only on apoptotic cells, but also on activated platelets and lymphocytes (10). In addition, some tumor cells constitutively expose PtdSer (19). These PtdSer-exposing live cells are not engulfed by macrophages, probably because the flippase is still active in them.

PtdSer-binding proteins and signal transducers for efferocytosis

Macrophages express soluble PtdSer-binding proteins or membrane-bound PtdSer receptors. In addition, there are serum PtdSer-binding proteins. Macrophages use these PtdSer-binding molecules to trap apoptotic cells and to execute the efferocytosis.

Protein S (ProS) and growth-arrest-specific 6 (Gas6) are serum proteins that share 40% identity in their amino acid sequences and have similar structural motifs (20). They specifically bind PtdSer at a Gla (gamma-carboxy glutamic acid) domain with similar affinities (21). Milk fat globule EGF factor 8 (MFG-E8) was originally discovered on the surface of milk fat globules in mammary glands and was later found to be secreted from certain macrophages and immature dendritic cells (22). MFG-E8 binds PtdSer at a Factor VIII homologous region with an affinity 5–10 times that of ProS/Gas6 (23). T-cell immunoglobulin and mucin domain-containing molecule 4 (Tim4) and its homologue Tim1 are type I transmembrane proteins expressed by resident macrophages and injured kidney, respectively (24, 25). These molecules bind PtdSer at an immunoglobulin (Ig) domain (24) with the same affinity as

MFG-E8 does. Thus, there are at least three classes of proteins (Gas6/ProS; Tim1/Tim4; and MFG-E8) that use different motifs to bind PtdSer.

The soluble proteins such as ProS/Gas6 and MFG-E8 cannot support efferocytosis by themselves. Even Tim4, a type I membrane protein, cannot support efferocytosis, because its cytoplasmic region is apparently too short (40 amino acids) to mediate the signals for efferocytosis (26). ProS and Ga6 preferentially bind to one of the TAM (Tyro3, Axl and MerTK) receptors (27–29), which have tyrosine kinase activity, whereas MFG-E8 binds to the $\alpha_v\beta_{3/5}$-integrin complex to transduce the signal (23). Macrophages express at least one of the TAM receptors (Axl or MerTK; or both in most cases) as well as the $\alpha_v\beta_3$-integrins. Thus, ProS/Gas6 and MFG-E8 can act as a bridge between PtdSer-exposing apoptotic cells and macrophages.

Tim4 is expressed on the resident macrophages in various tissues, including Kupffer cells, skin macrophages and marginal zone macrophages in the spleen (27, 30). Since Tim4's affinity for PtdSer is 4–8 times that of ProS/Gas6, it seems likely that apoptotic cells are first recruited to macrophages ('tethering') via Tim4 and then passed to the Gas6/ProS–TAM system for engulfment ('tickling'), at least in resident macrophages (Fig. 2). Tingible-body macrophages reside in the germinal centers of the spleen and lymph nodes, and have a strong capacity to engulf apoptotic cells, particularly activated B cells undergoing apoptosis. These macrophages express MerTK, MFG-E8, Tim4 (30–32) and probably integrin-$\alpha_v\beta_3$. However, how these molecules collaborate to elicit efferocytosis is still unclear.

Signal transduction for efferocytosis

As found in the phagocytosis of bacteria, actin on macrophages polymerizes during efferocytosis, forming actin patches that establish phagocytic cups (33). Apoptotic cells then sink into the macrophage via the phagocytic cups and are transported to lysosomes (34). These processes are accompanied by the activation and inactivation of Rho family proteins such as Rac1 (Fig. 2). Classical experiments in *Caenorhabditis elegans* showed that Rac1 and several of its associated molecules have roles in efferocytosis and these molecules were genetically characterized (35, 36). These include CED7 (ABC transporter), CED2 (Crk), CED5 (DOCK2) and CED10 (Rac1). However, how these molecules are activated by apoptotic cells during efferocytosis is not well understood. TAM-family receptor kinases and integrin-$\alpha_v\beta_3$, which are essential signal transducers for efferocytosis, activate a variety of signaling pathways (Src, Akt, NF-κB, JAK, Fak and others) (37, 38). However, how the activation of these molecules is integrated to form the phagocytic cup for efferocytosis remains a challenging research topic.

TAM receptor-mediated efferocytosis is known to stimulate the production of anti-inflammatory cytokines such as IL-10 and TGFβ by macrophages (5, 39). In addition, TAM receptors broadly inhibit toll-like receptor (TLR)-signaling cascades and the interferon receptor (IFNR)-signaling cascades (40), damping the inflammatory response. Thus, efferocytosis has been characterized as an anti-inflammatory process.

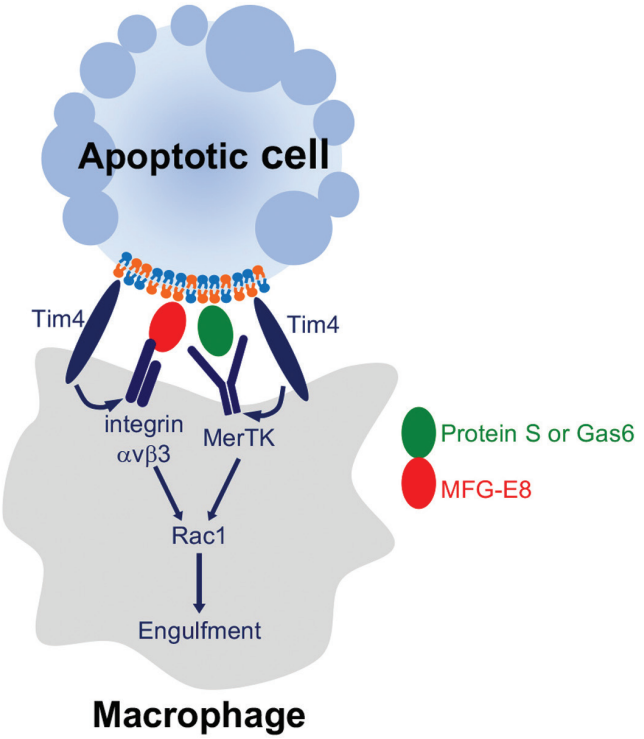

Fig. 2. Tethering and tickling processes in efferocytosis. In a set of resident macrophages, PtdSer-exposing apoptotic cells at first bind to Tim4 on the macrophages (the 'tethering' process). Captured apoptotic cells then bind to a TAM tyrosine kinase receptor (e.g. MerTK) with the help of ProS or Gas6. This induces phosphorylation of the TAM receptor and promotes efferocytosis by activating Rac1 (the 'tickling' process). In other types of macrophages and immature dendritic cells such as tingible-body macrophages and skin Langerhans cells, a secreted protein MFG-E8 functions as a bridge between PtdSer on apoptotic cells and integrins on macrophages for efferocytosis.

Defective apoptotic-cell clearance in autoimmune disease

PtdSer is exposed on the surface of apoptotic cells when their plasma membrane is apparently intact. Therefore, when the PtdSer-exposing apoptotic cells are engulfed by phagocytes, intracellular materials are not released from the dead cells. On the other hand, when apoptotic cells are not promptly engulfed by macrophages, they undergo secondary necrosis, in which the plasma membrane ruptures, releasing intracellular components (41). The released cellular materials act as 'damage-associated molecular patterns' (DAMPs) to stimulate inflammatory and immunogenic reactions, which are likely to trigger an autoimmune response (Fig. 3).

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects multiple organs, such as the skin, kidney, lungs and nervous system (42). Patients with SLE carry autoantibodies against cellular components such as nuclei [antinuclear antibodies (ANA)], DNA [anti-double-stranded DNA (dsDNA) or anti-single-stranded DNA (ssDNA) antibodies] and phospholipids (anti-phospholipid antibodies). SLE can be triggered by intrinsic or environmental stimuli (43). One of these intrinsic factors is excessive apoptosis, followed by the exposure of self-antigens released from cells that have

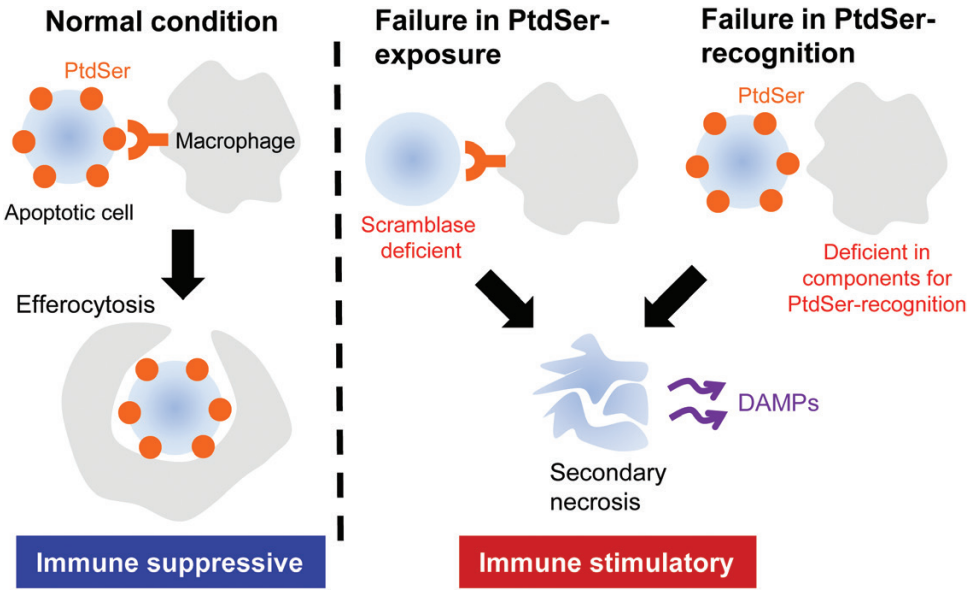

Fig. 3. Defects in efferocytosis and immunogenic response. Macrophages recognize PtdSer exposed on apoptotic cells, promptly engulf them (efferocytosis) and produce anti-inflammatory molecules (left) in normal conditions. Efferocytosis is thus anti-inflammatory. A deficiency of the Xkr8-scramblase causes delayed PtdSer exposure in apoptotic cells, leading to inefficient efferocytosis. A defect in the PtdSer-recognition system (Tim4, MFG-E8 and TAM receptor kinases) (right) also impairs efferocytosis. When the efferocytosis is impaired, dead cells undergo secondary necrosis and release DAMPs to stimulate inflammatory reactions.

undergone secondary necrosis (44, 45). Various lines of evidence indicate that apoptotic-cell clearance is defective in SLE patients (43). For example, monocyte-derived macrophages from SLE patients have a reduced capacity to carry out efferocytosis (46). In addition, apoptotic cells are reported to accumulate in the lymph-node germinal centers (47), bone marrow (48) and UV-irradiated epidermis of SLE patients (49).

Knocking out genes in mice that are essential for apoptotic-cell recognition causes defective efferocytosis and induces SLE-type autoimmune diseases. Abnormalities of molecules involved in efferocytosis have also been found in human SLE patients. Thus, SLE can now be discussed in molecular terms. As described above, MFG-E8 is a bridging molecule between apoptotic cells and macrophages. A lack or excess of MFG-E8 blocks efferocytosis *in vitro* (50). Accordingly, mice lacking MFG-E8 accumulate apoptotic B cells in the germinal centers and develop a lupus-like autoimmune disease (31). Administering recombinant MFG-E8 induces a similar autoimmune disease in mice (51), and a high level of MFG-E8 is found in the serum of human SLE patients (50, 52).

Similarly, *Mertk*-deficient mice, in which efferocytosis by macrophages is significantly delayed, develop high levels of auto-antibodies in the serum (53–55). A triple mutation in all TAM receptors (Tyro3, Axl and MerTK) exaggerates the autoimmune phenotype, with respect to the titer of auto-antibodies, number of activated lymphocytes and severity of arthritis (56). In addition, the extracellular region of TAM receptors can be shed by metalloproteinases and these soluble forms can inhibit efferocytosis by trapping ProS/Gas6 (57). A high level of the shed extracellular region of TAM receptors (soluble MerTK, Axl and Tyro3) is detected in the serum of human SLE patients (58, 59).

A similar story may apply to Tim4. T and B cells are hyperactivated in *Tim4*-deficient mice and these mice develop autoantibodies to dsDNA (60). Dual targeting of both the *Tim4* and *Mfge8* genes augments the autoimmune phenotype (61). Tim4 and Tim1 can be cleaved by metalloproteinase and the shed soluble region can bind PtdSer (62), suggesting that the soluble form of Tim4 may also trigger SLE.

Lupus-like autoimmune disease in *Xkr8*-deficient mice

As discussed above, defects in the PtdSer-recognition or efferocytosis system can trigger autoimmunity. It is possible that a defect in the PtdSer-exposing system has a similar effect. In fact, we recently found that mice that are defective in exposing PtdSer on apoptotic cells develop an SLE-type autoimmune disease (63).

Among the three Xkr members (Xkr4, Xkr8 and Xkr9) with apoptotic scramblase activity, mouse hematopoietic cells express only Xkr8. Notably, the thymocytes, splenocytes and neutrophils from *Xkr8*$^{-/-}$ mice show significantly delayed apoptotic PtdSer exposure and retarded PtdSer-dependent efferocytosis (63). These results confirmed that the Xkr8-mediated PtdSer exposure in apoptotic cells is essential for efficient efferocytosis by primary hematopoietic cells. Accordingly, *Xkr8*-deficient mice carry an increased number of TUNEL-positive (i.e. apoptotic) cells in the thymus at a young age (5 weeks old), and this phenotype is further pronounced by inducing apoptosis of the thymocytes with dexamethasone. Neutrophils have a short lifespan in the periphery (<10 h) and senescent neutrophils travel to the bone marrow, spleen or liver to be cleared by macrophages. Senescent neutrophils accumulate in the spleen of *Xkr8*-deficient mice (63), indicating that the caspase pathway leading to PtdSer exposure is required for the clearance of senescent neutrophils.

Xkr8-deficient mice do not develop a lupus-like phenotype in the C57BL/6 background. However, *Xkr8*-null female mice in the MRL background produce a large quantity of ANA and anti-dsDNA antibodies, and develop glomerulonephritis with a substantial deposition of immune complexes at the glomeruli (63).

Compensatory mechanisms that block autoimmune disease

As discussed above, when apoptotic cells are not swiftly engulfed, they undergo secondary necrosis and release DAMPs to activate the immune system. However, DAMPs released from the secondary necrotic cells alone are not sufficient for SLE development (44). The SLE phenotype in efferocytosis-defective murine models strongly depends on the mouse strain. Mice deficient in MFG-E8, Xkr8 or TAM receptors develop an autoimmune disease in a 129/B6 mixed or MRL background, but not in the C57BL/6 background (31, 63). There appear to be two mechanisms—the C1q-mediated clearance of dead cell components and the degradation of DAMP-type DNA by DNase—to prevent the development of autoimmunity (Fig. 4).

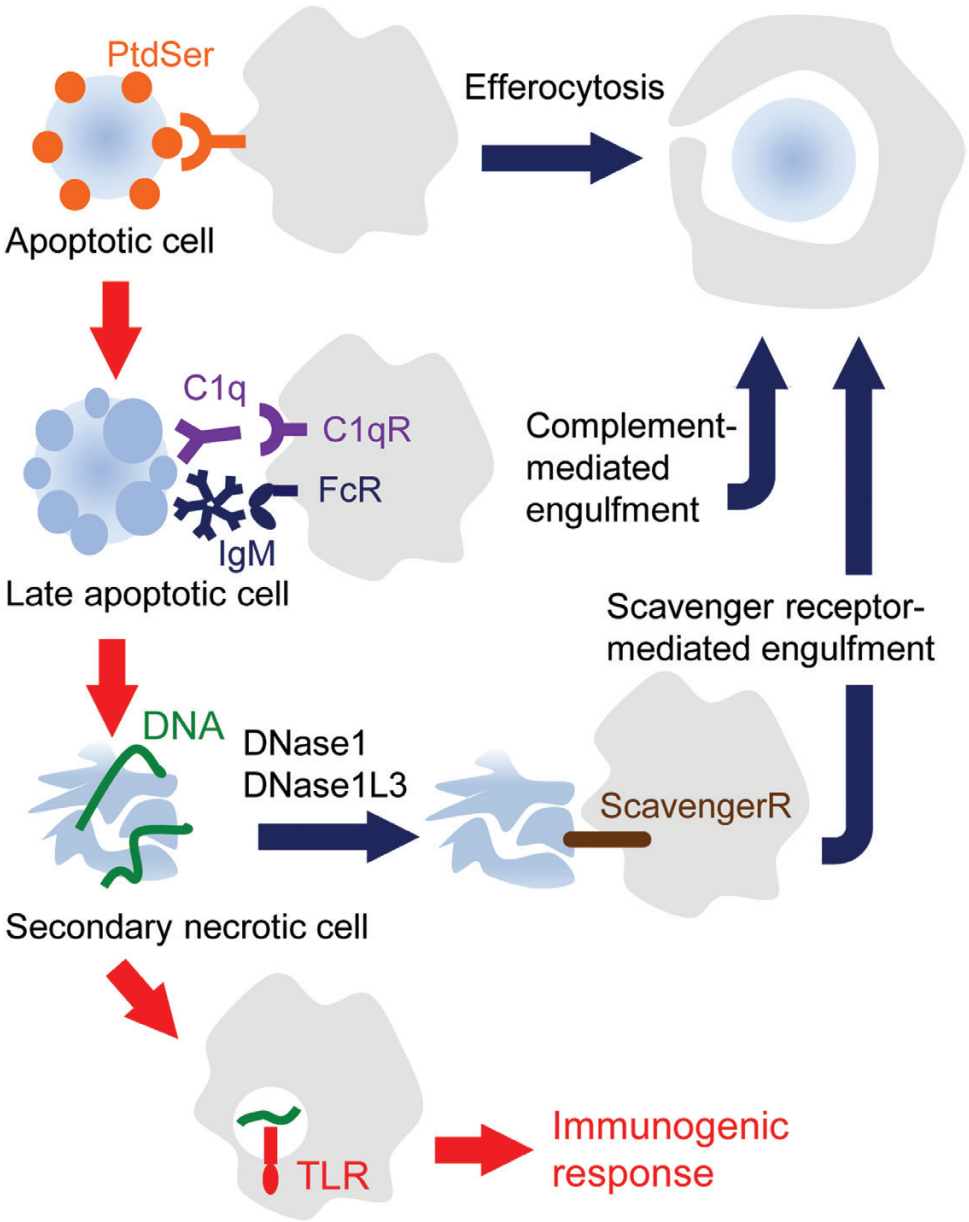

Fig. 4. Compensatory mechanisms for clearing dead cell components. Macrophages recognize PtdSer on apoptotic cells as an 'eat me' signal, and swiftly engulf them (efferocytosis). When apoptotic cells are not swiftly engulfed by efferocytosis, they can be engulfed by macrophages via the C1q receptor (C1qR) or Fc receptors (FcRs) at the late stage of apoptosis. Dead cells that escape from this engulfment then undergo necrosis and release various cellular contents through their ruptured plasma membrane. Chromosomal DNA released from the necrotic cells can be digested by DNase1 and DNase1L3, and the cellular remnants can be engulfed by phagocytes via scavenger receptors. If DNA is not efficiently cleaved by these DNases, DNA may aggregate the cellular debris to form strong antigens and/or stimulate macrophages or dendritic cells and trigger innate immunity via TLRs.

Although C1q, the first component of complement, was first reported to bind apoptotic cells and promote efferocytosis (64), recent reports indicate that it binds to cells at a late stage of apoptosis in a serum factor-dependent manner, suggesting that it is a back-up system for removing necrotic cells or DAMPs (65, 66). In addition, cellular components can be engulfed by phagocytes via scavenger receptors without inducing inflammation (67, 68). Patients with a C1q deficiency are rare, but they have the highest (>90%) prevalence of SLE (69). Furthermore, a *C1q* polymorphism that causes low serum C1q is associated with SLE (70). *C1q*-deficient mice carry excess apoptotic bodies in the glomerulus and develop a lupus-like phenotype (71). The disease severity in *C1q*$^{-/-}$ mice also largely depends on the genetic background: *C1q*$^{-/-}$ C57BL/6 mice exhibit no sign of autoimmune disease, whereas *C1q* deficiency in a 129 × C57BL/6 hybrid or MRL background induces an early onset and accelerated autoimmune disease (71, 72).

The recognition of nucleic acids is a mechanism for triggering an immune reaction (73). Many DNA sensors recognize not only pathological non-self DNA but also self DNA (74). Both chromosomal DNA and mitochondrial DNA can be released from cells that undergo secondary necrosis. Activated neutrophils also release DNA as 'neutrophil extracellular traps' (NETs) (75). Extracellular DNA is usually digested by the serum endonucleases DNase1 and DNase1-like 3 (DNase1L3, also called DNase γ). The DNase activities of these enzymes have similar biochemical characteristics except that DNase1 preferentially digests naked DNA, whereas DNase1L3 cleaves DNA that is associated with proteins (chromatin DNA or DNA in microparticles) (76, 77). Thus, when chromatin is released from dying necrotic cells, or released as microparticles from apoptotic cells, DNase1L3 is the major DNase that digests the DNA. DNase1 can cleave chromatin DNA only after the associated proteins have been degraded by proteases (77, 78).

A loss-of-function mutation of *DNase1* is associated with SLE in humans and mice (79, 80). In particular, the loss of DNase1 activity in the kidney appears to contribute to glomerulonephritis in the (NZB × NZW)F1 mouse (81). Similarly, loss-of-function mutations of the *DNase1L3* gene cause SLE in humans and mice (77, 82). Notably, the mouse MRL strain, in which a deficiency of Xkr8, MerTK or C1q induces the SLE phenotype, carries a missense mutation in the gene encoding DNase1L3 that reduces its ability to digest chromatin DNA (83). These results suggest that a deficiency in DNase1 or DNase1L3 increases the susceptibility of humans and mice to SLE.

Conclusions

Every day in our body, billions of cells undergo apoptosis and are swiftly removed by macrophages. This prompt clearance of apoptotic cells is essential to prevent the release of immunogenic materials from dying cells and thus the development of an autoimmune response. Current human data suggest that the accumulation of apoptotic cells owing to inefficient clearance is a causative event for SLE (43). Murine models with defective efferocytosis, such as MFG-E8-, Tim4-, TAM- or Xkr8-deficient mice, exhibit lupus-like phenotypes, supporting this hypothesis (31, 53–55, 60, 61, 63). Backup systems (complement or scavenger receptor systems) also exist to remove necrotic cells.

In addition, we now know that the accumulation of apoptotic cells, followed by the release of DAMPs from the necrotic cells alone is not sufficient to induce autoimmunity. Two DNases (DNase1 and DNase1L3) help to prevent the SLE phenotype by digesting the DNA released from dead cells. It should be noted that another DNase (DNase II) is involved in the digestion of the DNA of dying cells in lysosomes. A deficiency of DNase II causes the production of type I interferons and TNFα, and induces severe anemia and polyarthritis in mice and humans (84–86), confirming that the clearance of dead cell components, DNA in particular, is essential to maintain homeostasis in humans.

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