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Apoptosis and Clearance of Apoptotic Cells

Shigekazu Nagata

Laboratory of Biochemistry and Immunology, World Premier International Research Center Initiative Immunology Frontier Research Center, Osaka University, Osaka 565–0871, Japan; email: snagata@ifrec.osaka-u.ac.jp

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Abstract

The human body generates 10–100 billion cells every day, and the same number of cells die to maintain homeostasis in our body. Cells infected by bacteria or viruses also die. The cell death that occurs under physiological conditions mainly proceeds by apoptosis, which is a noninflammatory, or silent, process, while pathogen infection induces necroptosis or pyroptosis, which activates the immune system and causes inflammation. Dead cells generated by apoptosis are quickly engulfed by macrophages for degradation. Caspases are a large family of cysteine proteases that act in cascades. A cascade that leads to caspase 3 activation mediates apoptosis and is responsible for killing cells, recruiting macrophages, and presenting an "eat me" signal(s). When apoptotic cells are not efficiently engulfed by macrophages, they undergo secondary necrosis and release intracellular materials that represent a damage-associated molecular pattern, which may lead to a systemic lupus-like autoimmune disease.

INTRODUCTION

People die for various reasons—aging, infection, accidents, organ malfunction, suicide. We bury or cremate corpses swiftly to prevent the spread of pathogens. Cells, too, die for various reasons and are swiftly cleared by other cells acting as morticians.

The adult human body consists of 3.72×10^{13} cells of various types (70.1% erythrocytes, 6.8% endothelial cells, 5.0% skin fibroblasts, 8.0% glial cells, etc.) (1). When the body is shaped during embryogenesis, many cells are overproduced, and the excessive or harmful cells are developmentally programmed to undergo cell death (2). The dead cells are swiftly recognized and phagocytosed by tissue-resident macrophages. This process occurs in interdigit formation, sexual differentiation, neural-network formation, and immune-system development. Different cell types have different lifespans (for example, neutrophils, less than 10 h; enterocytes, 5–7 days; erythrocytes, 120 days; hepatocytes, 150 days; cortical neurons, the lifetime of the body) (3–6). Thus, several hundred billion cells die daily and are replaced by newly generated cells. How they die depends on their cell type. Senescent neutrophils die and are then phagocytosed by macrophages in the bone marrow, liver, and spleen (7, 8), while senescent red blood cells are recognized and phagocytosed by macrophages in the spleen and liver (9). Senescent enterocytes in the small intestine are shed into the lumen of the intestine (10). Cells infected by viruses or bacteria undergo cell autonomous necrosis or are killed by the immune system. These cells may not be recognized by macrophages as whole, and necrotic cells release materials that may activate the immune system. The inflammation caused by bacterial or viral infection involves a massive production of white blood cells, and when the infection ceases, these cells quickly die. In addition, various anticancer drugs kill tumor cells by inhibiting the synthesis of purines, DNA, or RNA or by cross-linking or intercalating DNA. In this review, I briefly describe the process of cell death and then discuss how dead cells are engulfed and cleared by macrophages.

PROGRAMMED CELL DEATH

Lockshin & Williams (11) originally defined programmed cell death in the context of insect development. Subsequently, Kerr et al. (12) noticed by ultrastructural analysis two morphologically different types of cell death in humans, apoptosis and necrosis. In necrosis, the cells swell, plasma membranes rupture, and cellular components are released; in apoptosis, the cells shrink with integral but ruffling plasma membranes, and nuclei are condensed and fragmented. Apoptotic cells are swiftly phagocytosed by macrophages to prevent the release of intracellular components. This process prevents the release of inflammatory factors and is thus called clean cell death. Since apoptosis occurs in developing embryos or in cells that die under physiological conditions, "programmed cell death" and "apoptosis" are often used synonymously. Furthermore, when apoptosis was discovered to be mediated by gene products, it was regarded as being programmed. Thus, the term programmed cell death has been used, confusingly, with two different meanings: the cell death programmed into animal development, and the cellular death process elicited by a molecular mechanism. Meanwhile, cell death with a necrotic morphology that occurs during inflammation or infection was also found to be programmed or regulated by gene products and was categorized as necroptosis and pyroptosis (13). In addition, nonapoptotic cell death was observed during *Caenorhabditis elegans* development (14) and *Drosophila* metamorphosis (15), indicating that cell death in animal development can occur by a nonapoptotic mechanism. Thus, programmed cell death should not be used as a synonym for apoptosis; it should be reserved for the cell death that takes place in animal development, as originally defined (11).

APOPTOSIS

Apoptosis plays an important role in animal development. For example, interdigital cells, nonfunctional nerve cells, and activated lymphocytes are removed by apoptosis. The involution of mammary glands is also mediated by apoptosis. In contrast, apoptosis may not play a major role in clearing senescent cells (red blood cells, and intestinal enterocytes). Apoptosis is mediated by specific sets of caspases that act in cascades, at the end of which caspase 3 or 7 is responsible for killing the cells. Apoptotic cells are engulfed by macrophages in a process called efferocytosis (16). Because efferocytosis is efficient and swift, it is difficult to find free apoptotic cells in vivo, even in tissues where large numbers of cells undergo apoptosis (17). Thus, apoptosis involves mechanisms not only for killing cells but also for recruiting macrophages ("find me") and presenting a signal(s) ("eat me") to the macrophages for cell engulfment (18, 19).

CASPASES

In the early 1990s, the Horvitz group (20) found that CED-3 (cell death abnormal 3), which is essential for programmed cell death in *C. elegans*, is homologous to mammalian ICE (IL-1β-converting enzyme), a protease responsible for producing mature IL-1β. The human genome project, started in 1990, revealed many genes homologous to ICE, some of which were found to be involved in apoptosis. Biochemical and cellular analyses indicated that these ICE homologs are proteases with a motif consisting of histidine and cysteine residues at the active site (21). These proteases recognize at least five amino acid residues on their target proteins and cleave peptide bonds strictly after aspartate (position P1). This protease family was called caspases, for cysteine-proteases that cleave after aspartic acid, or cysteine aspartyl proteases (22). Caspases are synthesized as precursors, or zymogens. Specific cleavages at two positions remove the prodomain at the N terminus and generate an active enzyme consisting of two large and two small subunits ($\alpha_2\beta_2$). There are 12 human and 11 mouse caspases. Some caspases have different recognition sequences (21), and those that recognize a certain sequence often cleave different substrates with different efficiency (23), indicating that the substrate specificity of caspases is determined by both the target sequence and the tertiary structure of the substrate. One group of caspases (human caspases 2, 3, and 6–10) mediates apoptosis, while another group (human caspases 1, 4, and 5) mediates pyroptosis, which causes inflammation. Consistent with these distinct caspase groups, the proteins cleaved during inflammation are different from those cleaved during apoptosis (24).

INTRINSIC DEATH PATHWAYS

Two apoptosis pathways, intrinsic and extrinsic, have been identified in mammalian systems (25) (**Figure 1**). The intrinsic pathway, also called the mitochondrial pathway, operates in developmentally controlled and genotoxic agent–mediated apoptosis and is regulated by Bcl-2 family members. The Bcl-2 family consists of three subfamilies: proapoptotic BH3-only members (Bim, Bid, Puma, Noxa, Hrk, Bmf, and Bad), proapoptotic effector molecules (Bax and Bak), and antiapoptotic Bcl-2 family proteins (Bcl-2, Bcl-xL, Mcl1, A1, and Bcl-B) (26). In healthy cells, the Bax/Bak action is arrested by antiapoptotic Bcl-2 family members. In response to an apoptotic stimulus (developmental cue, lack of growth or survival factor, or genotoxic agent), BH3-only members are transcriptionally or posttranscriptionally upregulated. The activated BH3-only proteins act on Bak and Bax or antagonize the antiapoptotic Bcl-2 family members. Bax and Bak then stimulate the release of cytochrome *c* from mitochondria. Cytochrome *c*, together with Apaf-1, forms a heptametrical complex called an apoptosome in a dATP/ATP-dependent manner (27).

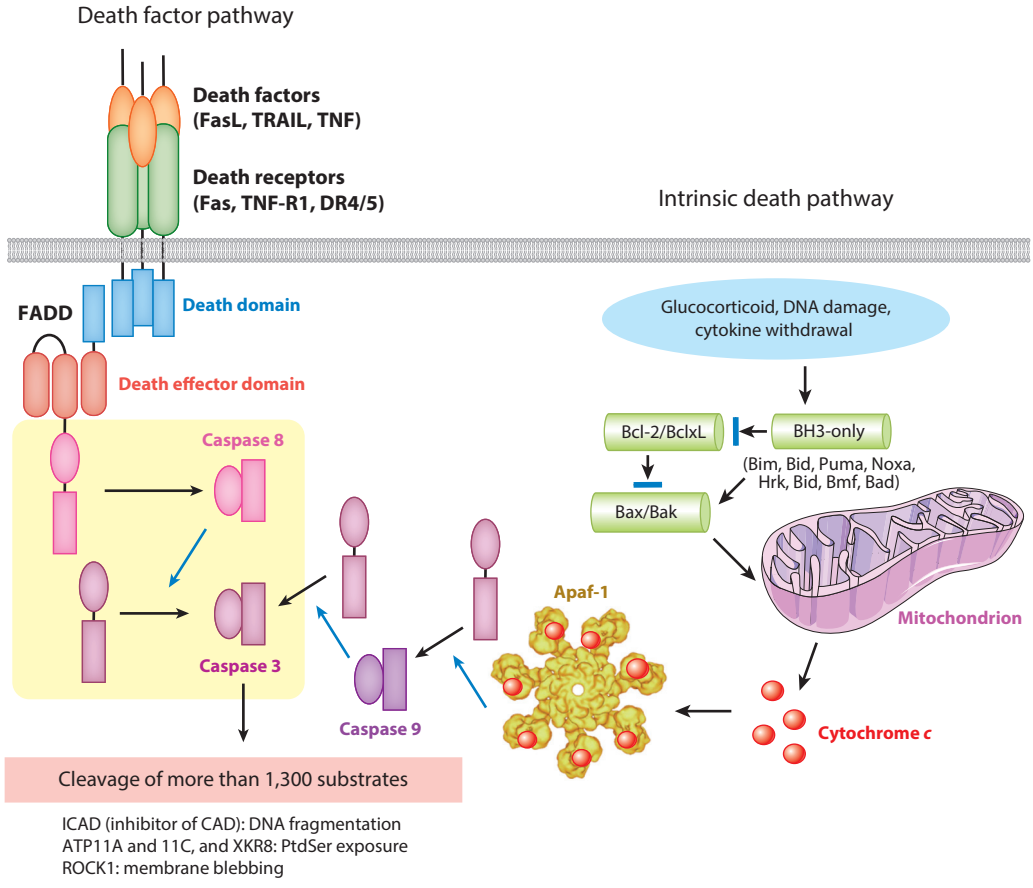

Figure 1

Signal transduction of apoptosis. Two distinct apoptotic signaling pathways converge. The death factor pathway, or extrinsic death pathway, is activated by death factors such as FasL, TNF-α, and TRAIL. Binding of a death factor to its specific receptor generates DISC (death-inducing signaling complex) and causes processing of procaspase 8 into mature active caspase 8. DISC is formed via homologous associations between death domains of Fas and FADD (*blue*) and between death effector domains (DEDs) of FADD and procaspase 8 (*orange*). Procaspase 8 carries two DEDs that are self-associated. In another pathway triggered by genotoxic agents, cytokine withdrawal, or developmental clues, BH3-only proteins (Bim, Bid, Bad, Bmf, Hrk, Puma, and Noxa) are activated transcriptionally or posttranscriptionally. They directly (or indirectly, by antagonizing Bcl-2) induce Bax/Bak-oligomerization, and the oligomerized Bax/Bak acts on mitochondria to release cytochrome *c*. The cytochrome *c* binds APAF-1 to form a heptameric complex that acts as a scaffold to mediate the conversion of procaspase 9 to active caspase 9. The caspase 8 activated in the extrinsic pathway and caspase 9 in the intrinsic pathway cleave procaspase 3 to mature caspase 3, which cleaves more than 1,300 cellular substrates to execute the apoptosis. For example, ICAD (inhibitor of caspase-activated DNase) is cleaved to release CAD (caspase-activated DNase) for apoptotic DNA fragmentation (241), ATP11A/11C and XKR8 for PtdSer exposure (**Figure 2**), and ROCK1 for apoptotic membrane blebbing (242). Black arrows indicate direct conversion, while blue arrows indicate catalytic actions. Figure modified from References 29 and 243.

The X-ray structure of the apoptosome, together with the biochemical analysis of its assembly, revealed that Apaf-1 exists in an autoinhibitory form in a complex with ADP (28). The binding of cytochrome *c* to Apaf-1 replaces the ADP with dATP (ATP), and the dATP (ATP)-bound Apaf-1 forms a heptameric complex with cytochrome *c*. This complex recruits monomeric procaspase 9 and helps it to form a dimer, which then undergoes autocatalytic cleavage to form an active heterotetrameric complex. The activated caspase 9 then cleaves and activates caspase 3. Although

cytochrome *c* is not required for the apoptosome formation in nematodes and flies (27), the other molecules (Bcl-2, Apaf-1, and caspase) are required for apoptosis in these organisms, suggesting that the intrinsic death pathway is conserved in metazoans.

THE DEATH RECEPTOR PATHWAY

Death Factors

The extrinsic apoptosis pathway is triggered by death factors of the TNF family (FasL, Fas ligand; TNF-α, tumor necrosis factor α; TRAIL, TNF-related apoptosis-inducing ligand) (29–31). These factors are synthesized as type II membrane proteins with a homotrimeric structure and can be cleaved from the membrane to generate a soluble form. TNF-α is predominantly cleaved and shed by a metalloprotease called ADAM17 (a disintegrin and metalloprotease) or TACE (TNF-α-converting enzyme), while FasL is cleaved by ADAM10 (32, 33).

FasL's apoptosis-inducing activity is executed by its membrane-bound form (34–36). The soluble trimeric form of FasL generally acts as an antagonist against the membrane-bound FasL in vitro and in vivo (35–37). Fusing a Flag tag and leucine (or isoleucine)-zipper motif (LZ-FasL) (38, 39) or HA-Flag-ACRP30 [the trimeric domain of ACRP30, a complement factor (C1q) homolog] (Mega-FasL) (40) to the N terminus of soluble FasL generates a hexameric FasL, which dramatically gains apoptosis-inducing activity. These findings agreed with a previous observation that cross-linking Apo1 (Fas) to an agonistic anti-Apo1 antibody strengthens its killing activity (41), and confirmed that a high-order oligomeric structure is necessary for efficient FasL-induced death signaling.

Note that there are three types of soluble FasL in the field: one corresponding to the soluble form generated by shedding from membrane-bound FasL, one engineered to form an oligomer (LZ-FasL or ACRP-FasL), and one secreted as an exosome-bound form. The shed form of FasL is commercially available and usually acts as an antagonist against membrane-bound FasL (35), although it can induce apoptosis in cells that are very sensitive to Fas-mediated apoptosis, for example, due to a high expression level of Fas (42). In contrast, LZ-FasL, ACRP-FasL, and exosome-bound FasL appear to mimic membrane-bound FasL (38, 43).

Death Receptors

The specific receptor for FasL is a type I membrane protein called Fas (also called APO-1 or CD95) belonging to the TNF receptor family. The cytoplasmic region of Fas, as well as those of TNF receptor 1 (TNF-R1), TRAIL-R1 (DR4), and TRAIL-R2 (DR5), contains an approximately 80–amino acid region called the death domain that is required for death signaling (29, 30, 44). The oligomerized Fas death domain recruits FADD (Fas-associated protein with death domain), procaspase 8, and a procaspase 8–like protein called c-FLIP (cellular FLICE/caspase 8 inhibitory protein; FLICE stands for FADD-like ICE) (29, 30, 44). FADD carries a death domain and a death effector domain, while procaspase 8 and c-FLIP contain two death effector domains at their N terminus, by which they associate with FADD. The complex consisting of Fas, FADD, procaspase 8, and c-FLIP is called DISC (death-inducing signaling complex) (45). Structure and stoichiometric analyses of the DISC components indicated that Fas, FADD, and procaspase 8 are present in the DISC at a ratio of 1:1:≥8 (46, 47).

Death factor–induced apoptosis signaling is proposed to branch into two pathways downstream of DISC. In type I cells (for example, thymocytes), where caspase 8 is strongly or sufficiently activated at DISC, caspase 8 directly activates procaspase 3. In type II cells (hepatocytes and

fibroblasts), caspase 8 cleaves Bid, a BH3-only member of the Bcl-2 family, and the cleaved Bid (tBid) induces cytochrome *c* release from mitochondria (31, 44). Cytochrome *c* then activates the caspase 9–caspase 3 pathway, as described above for the intrinsic death pathway. The presence or absence of XIAP (X chromosome–linked inhibitor of apoptosis protein) was proposed to distinguish type I and type II cells (48). However, a hexameric FasL (MegaFasL) or exosome-bound FasL induces apoptosis in hepatocytes without using the mitochondrial pathway, or directly activates caspase 3 via caspase 8 (43, 49). These results indicate that membrane-bound FasL, the natural ligand for Fas, does not discriminate between type I and type II cells and that FasL-induced apoptosis is determined by a balance between the strength of the death signal and the cell's sensitivity to the death signal.

Several groups recently reported that the Fas system not only activates apoptotic signaling but also transduces signals for cell proliferation or cytokine production (50, 51). Most of these studies used an agonistic anti-Fas antibody or soluble Fas ligand, which have only weak killing activity. The growth-stimulating action of FasL needs to be confirmed using membrane-bound FasL on activated T cells, or at least molecules with similar activity as membrane-bound FasL (MegaFasL, LZ-FasL, or exosome-bound FasL).

HALLMARKS OF APOPTOSIS, AND CASPASE SUBSTRATES

Apoptosis was originally defined by the characteristic morphology of the dying cells (12). Cells undergoing apoptosis shrink. Their plasma membrane is apparently intact, but it undergoes blebbing. The fragmentation of chromosomal DNA into nucleosomal units (52), exposure of phosphatidylserine (PtdSer) on the cell surface (53), and loss of mitochondrial potential (54) are biochemical hallmarks of apoptosis. These features disappear when apoptosis is induced in the presence of caspase inhibitors, indicating that they are all mediated by activated caspases. During the apoptosis of a human cell, nearly 1,300 different proteins are cleaved by caspases at more than 1,700 sites (55). Different caspases have preferred substrates (23). Since caspase 3 is at the end of the caspase cascade and is activated by both the intrinsic and extrinsic death pathways, it is conceivable that the general characteristics of apoptosis are mediated by the caspase 3 substrates. In fact, several apoptotic characteristics, including membrane blebbing (56), DNA fragmentation (57), and PtdSer exposure (58, 59) are mediated by caspase 3 targets (**Figure 1**). The PtdSer exposure is essential for apoptotic cells to be eaten, and I next discuss this process in detail.

ASYMMETRICAL DISTRIBUTION OF PHOSPHOLIPIDS

Flippases

Biological membranes consist of two layers, outer and inner leaflets, between which lipids are asymmetrically distributed (60). In the plasma membrane of eukaryotic cells, amine-containing or anionic phospholipids [PtdSer, phosphatidylethanolamine (PtdEtn), phosphoinositides (PtdIns), phosphatidic acids (PtdOH)] are predominantly or exclusively localized to the inner leaflet, whereas the outer leaflet is enriched in choline-containing phospholipids [phosphatidylcholine (PtdCho) and sphingomyelin (Sph)] and glycosphingolipids. Three types of lipid transporters have been proposed, based on the direction of transport, substrate specificity, and ATP requirement (61). Using the energy of ATP, flippases and floppases translocate specific lipids from the outer to inner leaflet or from the inner to outer leaflet, respectively, against a concentration gradient, whereas scramblases, driven by the existing lipid gradient, nonspecifically and bidirectionally transport lipids between the inner and outer leaflets.

The flippases that maintain the asymmetrical distribution of PtdSer are subfamily P4 of the P-type ATPases (62). P-type ATPases carry ten transmembrane segments and a large cytoplasmic domain with ATPase activity. They are called P type, because a transient phosphorylated intermediate is formed at a conserved aspartic acid during its catalytic cycle (63). Among the 14 P4 ATPase members, ATP11A and ATP11C are ubiquitously expressed, localized to the plasma membrane, and translocate PtdSer and PtdEtn from the outer to inner leaflet to confine them to the inner leaflet (59, 64). Like many other P4-type ATPases, ATP11A and ATP11C require CDC50A [also called transmembrane protein 30A (TMEM30A)] as a chaperone for their localization to the plasma membrane. The deficiency of both ATP11A and ATP11C in a mouse lymphoma cell line severely decreases the flippase activity, but the asymmetrical PtdSer distribution is still maintained. However, cells lacking CDC50A completely lose their flippase activity, and constitutively expose PtdSer on the cell surface, suggesting that other P4-type ATPases or molecules contribute to maintain the asymmetrical distribution of PtdSer.

PtdSer Exposure During Apoptosis

Cells undergoing apoptosis expose PtdSer on their surface in a caspase-dependent manner (53, 65). The cellular protein annexin V specifically binds to PtdSer, and fluorescently labeled annexin V is widely used to detect apoptotic cells (66).

Two and three caspase-recognition sites are present in the cytoplasmic region of ATP11A and ATP11C, respectively, in humans and mice, and their cleavage by caspase 3 inactivates their flippase activity (59, 64). However, flippase inactivation alone is insufficient to quickly expose PtdSer on the cell surface. PtdSer, like other phospholipids, consists of a polar head group and a hydrophobic tail region, and it does not easily translocate from the inner to the outer leaflet through the hydrophobic lipid layers. Thus, once the asymmetrical distribution of phospholipids is established, the rate of their spontaneous translocation or scrambling is extremely low (67, 68). Thus, an enzyme(s) or scramblase(s) is needed to perform this job.

PHOSPHOLIPID SCRAMBLASES

PLSCR

PtdSer is exposed not only when cells undergo apoptosis but also when platelets and erythrocytes are activated. The PtdSer exposure in activated platelets and erythrocytes occurs in a Ca^{2+}-dependent manner, and the exposed PtdSer functions as a scaffold for blood clotting factors. Comfurius et al. (69) and Basse et al. (70) established an assay system for phospholipid scramblase in 1996. They prepared membrane proteins from human platelets or erythrocytes and measured the phospholipid scrambling activity in reconstituted proteoliposomes. Using this system, a 37-kDa protein with Ca^{2+}-dependent scramblase activity was purified from human erythrocytes (70), and its cDNA was molecularly cloned. The 37-kDa protein, called phospholipid scramblase, or PLSCR1, belongs to a phylogenetically well-conserved gene family, and the PLSCR family has four members present in humans and mice (71). PLSCR1 is an endofacial plasma membrane protein containing one transmembrane domain at its C terminus. It is highly palmitoylated, which is essential for its Ca^{2+}-phospholipid scramblase activity in a cell-free system (72). However, subsequent studies with *PLSCR1*-deficient cells contradicted its role as a scramblase. *PLSCR1*-deficient erythrocytes and platelets responded normally to a Ca^{2+} ionophore to expose PtdSer (73). *PLSCR1*-null mice showed no hematology abnormalities but were defective in granulocyte colony–stimulating factor (G-CSF)-induced granulopoiesis (73). The *PLSCR1* gene is induced

by interferon, and interferon's antiviral activity was severely reduced in *PLSCR1*-deficient cells (74). PLSCR1 translocates to the nucleus and was found to be a membrane-tethered transcription factor of the tubby-like protein family (75). These results collectively indicated that PLSCR1's phospholipid-scrambling activity in reconstituted proteoliposomes (70, 76, 77) may not reflect its physiological activity, and that PLSCR is a misnomer.

TMEM16 Family

We found that Ca^{2+}-ionophore-induced exposure of PtdSer in mouse lymphoma cells is reversible under low-Ca^{2+} conditions. By repeated sorting for the top <5% of the population that exposed PtdSer (19 times), we established a subline of mouse lymphoma cells that strongly exposes PtdSer at low Ca^{2+}-ionophore concentrations (78). Expression cloning from a cDNA library of this cell line identified two different cDNAs that regulate phospholipid scrambling at plasma membranes; these cDNAs encode membrane proteins TMEM16F and XKR8 (58, 78).

Human and mouse TMEM16F, also called anoctamin 6, consists of 911 amino acids and was originally thought to carry 8 transmembrane segments with cytoplasmic N and C termini. TMEM16F belongs to the TMEM16 family, which has 10 members (TMEM16A–16H, 16J, and 16K) (79, 80). Except for TMEM16E, 16H, and 16K, which are intracellularly localized, the TMEM16 members are localized to the plasma membrane. TMEM16A and 16B are Ca^{2+}-activated Cl^- channels (79, 80), while TMEM16C, 16D, 16F, 16G, and 16J are Ca^{2+}-activated phospholipid scramblases (81). A TMEM16 homolog is present in the fungus *Nectria haematococca*, and X-ray structural analysis indicated that it carries 10 transmembrane segments instead of 8 and forms a homodimer (82), suggesting that mammalian TMEM16 also carries 10 transmembrane segments and forms a dimer.

Ca^{2+} directly binds to the TMEM16 dimer at acidic residues present in the transmembrane segments (82–84) and stabilizes its complex structure (84). A 35–amino acid region spanning the fourth to fifth transmembrane segments not only of TMEM16C, 16D, 16F, and 16J but also of TMEM16E and 16K confers the scrambling activity to TMEM16A (85–87), suggesting that TMEM16E and 16K function as phospholipid scramblases at the endoplasmic reticulum. A mathematical analysis of TMEM16-mediated phospholipid scrambling suggested that charged residues at the transmembrane segments of TMEM16 proteins act like stepping stones for the phospholipids being translocated (88). This theory was recently supported by a mutational analysis (87).

Among the TMEM16 members that have scramblase activity at the plasma membrane, TMEM16F is ubiquitously expressed, while others are expressed specifically in the brain (TMEM16C and 16D) or intestines (TMEM16G and 16J) (81). Lymphocytes, platelets, and embryonic fibroblasts express only TMEM16F among the scrambling-potent TMEM16 members, and a TMEM16F loss-of-function mutation causes defective PtdSer exposure in activated platelets, leading to inefficient blood clotting in humans (Scott syndrome), dogs, and mice (78, 89, 90).

Since Ca^{2+} increases during apoptotic cell death and regulates various apoptotic processes (91), Ca^{2+}-dependent scramblase was proposed to play a role in apoptotic PtdSer exposure. However, TMEM16F-null lymphoma cells, embryonal fibroblasts, and platelets, which were completely unable to expose PtdSer upon stimulation with Ca^{2+} ionophore, were fully able to expose PtdSer by means of apoptotic signals (81, 92), indicating that TMEM16F is not involved in apoptotic PtdSer exposure, or that another system compensates for its role in this exposure. This finding agreed with previous reports (93, 94) showing that the apoptotic PtdSer exposure and Ca^{2+}-induced PtdSer exposure can be mediated by two distinct mechanisms.

XKR Family

Mouse XKR8, the other cDNA identified as a phospholipid scramblase by expression cloning, encodes a 401–amino acid polypeptide (58). Analysis using the PredictProtein program (**https://www.predictprotein.org/**) indicated that XKR8 carries 10 transmembrane segments with cytoplasmic N and C termini. XKR8 belongs to the XK-related (XKR) protein family, which has 9 members in humans (XKR1–9). The founding member of the family, XK or XKR1, is localized to the plasma membrane of red blood cells and regulates divalent cation transport (95). XK is associated with Kell, a 95-kDa type II membrane protein with zinc endopeptidase activity (96). Kell is highly polymorphic, and the XK-Kell complex is a known blood antigen. A defect in *XK* causes McLeod syndrome, which is characterized by hemolytic anemia, myopathy, and neurological disorders (96).

XKR8 is expressed ubiquitously and is associated 1:1 with basigin or neuroplastin. Basigin and neuroplastin are type I membrane proteins belonging to the immunoglobulin superfamily (97). Basigin or neuroplastin functions as a chaperone to transport XKR8 to the plasma membrane from the endoplasmic reticulum. XKR8 carries a caspase 3/7-recognition motif at its C terminus and is cleaved by caspase 3 when cells undergo apoptosis (58). The cleaved XKR8 appears to dimerize to execute scramblase activity. Among other members of the XKR family, XKR4 and XKR9 carry a caspase 3–recognition site in their C terminus and can elicit apoptotic PtdSer exposure in *Xkr8*$^{-/-}$ cells (98). XKR4 and XKR9 are tissue-specifically expressed in the brain and intestine, respectively. Whether these molecules have a redundant role for apoptotic PtdSer exposure with XKR8, or have specific roles in their respective tissues is unknown. Both TMEM16F and XKR8 appear to carry 10 transmembrane segments, and their dimeric form acts as a phospholipid scramblase (97). However, whether the above-mentioned stepping stone model proposed for TMEM16 (87, 88) also applies to XKR8-mediated phospholipid scrambling remains to be studied.

PHOSPHATIDYLSERINE AS AN "EAT ME" SIGNAL

Macrophages engulf apoptotic or senescent cells but not healthy cells, suggesting that the engulfed cells present a determinant(s) or eat me signal on their cell surface. In 1982, Schroit & Fidler (99) showed that liposomes prepared with a mixture of phospholipids containing PtdSer bound more efficiently to, and were phagocytosed, by mouse alveolar macrophages in vitro. Schroit's group then showed that red blood cells loaded with nitrobenzoxadiazole-PtdSer (NBD-PS) were efficiently phagocytosed in vitro by primary macrophages (100) and in vivo by reticuloendothelial systems (Kupffer cells and macrophages) in the liver and spleen (101). NBD-PS serves as a substrate for flippase, and exogenously added NBD-PS is internally translocated (59, 102). However, the polar nature of NBD distorts its alignment in the membrane bilayers, slowing the efficiency of its translocation. Thus, a substantial amount of NBD-PS added to red blood cells was still localized to the outer leaflet of the plasma membrane (101). Since the loading of NBD-phosphatidylcholine or NBD-phosphatidylglycerol had little effect on engulfment of red blood cells by macrophages, PtdSer was proposed to be exposed on the surface of effete erythrocytes to serve as a signal triggering their phagocytosis (101).

After these findings, Fadok et al. (53) reported that PtdSer is exposed on the surface of apoptotic cells. Masking PtdSer with PtdSer-containing liposomes (53) or by PtdSer-binding proteins such as annexin V (103) or milk fat globule EGF factor 8 (MFG-E8) (104) inhibited the apoptotic cell engulfment by macrophages in vitro and in vivo. These results established PtdSer as a likely eat me signal from apoptotic cells to macrophages. As described above, PtdSer is confined to the inner leaflet of the plasma membrane in healthy cells by the action of flippases ATP11A and ATP11C

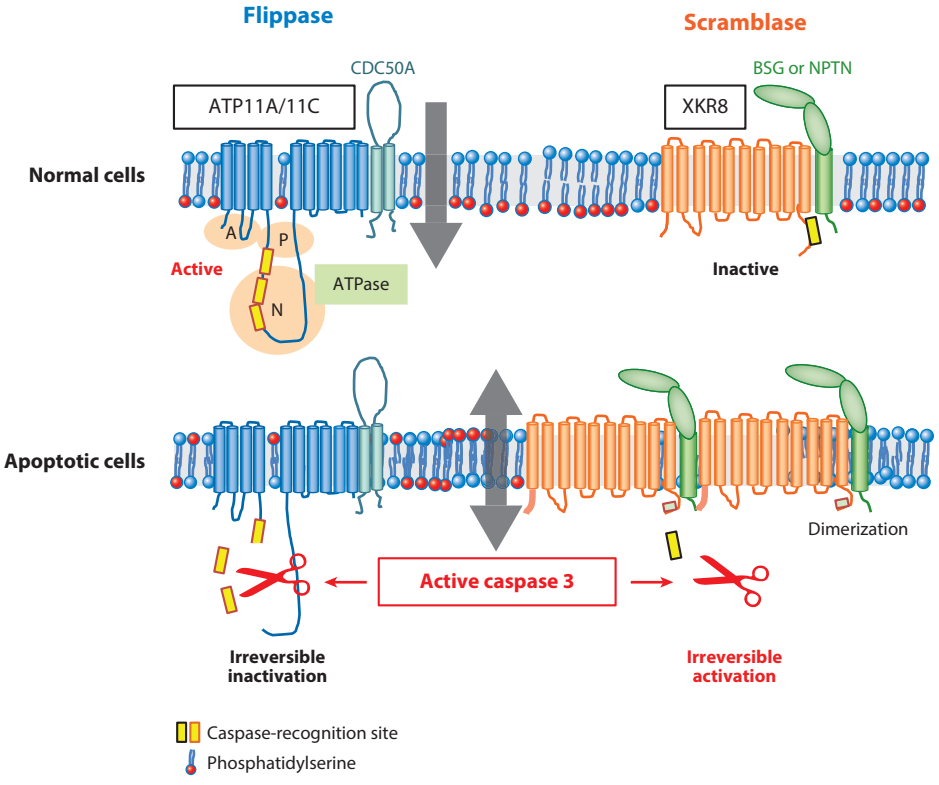

Figure 2

Molecular mechanism for apoptotic PtdSer exposure. ATP11A and ATP11C are P4-type ATPases that contain ten transmembrane segments. Their cytoplasmic region is divided into actuator (A), nucleotide-binding (N), and phosphorylation (P) domains and carries three (ATP11C) or two (ATP11A) caspase-recognition sites in the N domain. In healthy cells, ATP11A and ATP11C, together with their chaperone CDC50A, are present as a heterodimer at plasma membranes and work as flippase to translocate PtdSer from the outer to the inner leaflets. XKR8 is a member of the XK family that carries ten transmembrane regions and is complexed with basigin (BSG) or neuroplastin (NPTN), which work as a chaperone for XKR8 to translocate it to the plasma membrane. When cells undergo apoptosis, caspase 3 cleaves ATP11A and ATP11C to irreversibly inactivate them. Caspase 3 also cleaves XKR8 at the C-terminal tail region, and the truncated XKR8 together with BSG or NPTN undergoes dimerization. The dimerized XKR8-BSG/NPTN complex now works as a scramblase and nonspecifically and bidirectionally translocates phospholipids between the membrane bilayers, thus exposing PtdSer on the surface. Figure modified from Reference 122.

(59, 64). When cells undergo apoptosis, activated caspase 3 cleaves and inactivates ATP11A and ATP11C (59, 64). Concurrently, caspase 3 cleaves and activates XKR8 scramblase, to quickly expose PtdSer on the dead-cell surface (58) (**Figure 2**). Living cells expose PtdSer when they are transformed with a constitutively active form of TMEM16F (78), or when flippases are fully inactivated by a lack of CDC50A (59). Living cells that expose PtdSer by the lack of flippases but not by the constitutively active TMEM16F are engulfed by thioglycollate-elicited macrophages (59, 105), suggesting that the constitutive flipping of PtdSer in the latter case inhibits the engulfment of apoptotic cells by macrophages.

The PtdSer eat me system for apoptotic-cell engulfment is well conserved from *C. elegans* to humans. TAT1 and TAT3, the nematode P4-type ATPases responsible for maintaining the

asymmetrical distribution of phospholipids at plasma membranes (106), carry caspase-recognition sites in the middle of the molecule (107). CED8, an XKR8 homolog in *C. elegans*, functions as a phospholipid scramblase to expose PtdSer on the cell surface in a CED3 (caspase)-dependent manner (58, 108).

ENGULFMENT OF APOPTOTIC CELLS

deCathelineau & Henson (16) coined efferocytosis, Latin for "carry to grave," to describe the engulfment of apoptotic cells. Masking PtdSer inhibits efferocytosis (53, 103, 104), while the constitutive exposure of PtdSer due to a lack of flippase causes the efferocytosis of living cells (59), indicating that PtdSer exposure is necessary and sufficient as an eat me signal. Thus, many groups sought to identify molecules that recognize PtdSer, and two kinds were found: secreted soluble proteins, including MFG-E8, Gas6, and Protein S (PROS), and type I membrane proteins expressed in phagocytes, including TIM1, TIM4, and CD300. In this section, I discuss the assay systems for efferocytosis and the molecules proposed to recognize PtdSer in this process.

Assay for Efferocytosis

If apoptotic cells are not swiftly engulfed by phagocytes, they undergo gasdermin E– or DFNA5 (deafness, autosomal dominant 5)-mediated secondary necrosis (109, 110). To avoid this situation, prey for the efferocytosis assay should be apoptotic as synchronously as possible. Treating mouse thymocytes with FasL or dexamethasone usually provides good prey with apoptotic characteristics. It is also essential to distinguish efferocytosis (internalization of dead cells) from the binding of apoptotic cells to phagocytes. We used the knowledge that DNA is not fragmented during apoptosis in *CAD* (caspase-activated DNase)-deficient cells but is cleaved by DNase II in macrophages after being engulfed (111). Thus, efferocytosis could be quantified by incubating macrophages with *CAD*-deficient apoptotic thymocytes and then detecting TUNEL-stained phagocytes by flow cytometry (112). Later, Miksa et al. (113) developed a more convenient assay for efferocytosis in which the prey is labeled with pHrodo, a pH-sensitive fluorescent dye that emits fluorescence only under acidic conditions, after phagocytosis.

MOLECULES RECOGNIZING PTDSER FOR EFFEROCYTOSIS

PSR

In 2000, Fadok et al. (114) established a monoclonal antibody that inhibits PtdSer-dependent efferocytosis and used a phage-display method to identify the molecule recognized by the antibody. This led to the identification of an apparent type II membrane protein designated PtdSer receptor, or PSR (114). Mutations of *PSR* were reported to cause a slight delay in clearing dead cells in *C. elegans* (115). *PSR* knockout mice were embryonic lethal, which was interpreted to be due to the inefficient efferocytosis (116). Soon afterward, Bose et al. (117) established another *PSR*-deficient mouse line, and their careful studies showed that PSR is essential for the development of various organs during mouse embryogenesis, but not for the clearance of apoptotic cells. In fact, PSR was found to be identical to a nuclear protein called JMJD6 (Jumonji domain–containing protein 6) (118), which is involved in histone demethylation and mRNA splicing (119). Thus, PSR is apparently a misnomer (120–123); but, unfortunately, it is still cited as a PtdSer-recognizing molecule for efferocytosis (124, 125).

MFG-E8

To identify macrophage molecules involved in efferocytosis, we established a library of monoclonal antibodies against mouse thioglycollate-elicited peritoneal macrophages (112). Screening this library identified one monoclonal antibody that significantly enhanced efferocytosis. Its antigen was purified by affinity chromatography and found to be MFG-E8, a 440–amino acid molecule that stimulates efferocytosis. MFG-E8's name comes from its being a major component of milk fat globules and carrying two EGF domains at its N terminus and two factor VIII–homologous domains (C1 and C2) at its C terminus (126). One of the EGF domains contains an Arg-Gly-Asp (RGD) motif, through which MFG-E8 binds to the integrin-$\alpha_v\beta_3$ or integrin-$\alpha_v\beta_5$ complex in phagocytes. MFG-E8 also specifically binds PtdSer via its factor VIII–homologous domains with high affinity (K_D of approximately 2 nM), thus serving as a bridging molecule between PtdSer-exposing apoptotic cells and integrin-expressing phagocytes, for efferocytosis (112). MFG-E8 is expressed in a subset of phagocytes, including thioglycollate-elicited peritoneal macrophages (112), tingible-body macrophages in germinal centers (127), and GM-CSF-induced bone marrow–derived immature dendritic cells (128), and contributes to their ability to engulf apoptotic cells. MFG-E8 is strongly expressed by epithelial cells and macrophages in mammary glands that undergo involution and is involved in clearing the excess milk fat globules at the end of lactation (129). MFG-E8 is also upregulated in cardiac myofibroblasts during myocardial infarction, where it promotes efficient dead-cell engulfment to minimize inflammation and limit the damaged area (130).

Because MFG-E8 is a bridging molecule between apoptotic cells and phagocytes, the dose-dependency of efferocytosis on MFG-E8 is bell shaped (131), and either a lack or an excess of MFG-E8 causes various autoimmune diseases. *Mfge8*-null mice on a 129/B6 mixed background accumulate unengulfed apoptotic cells in germinal centers and develop systemic lupus erythematosus (SLE)-type autoimmune diseases (127). Unengulfed milk fat globules due to *Mfge8* deficiency during involution cause mastitis in mice (129). The same mutation on a C57/B6 background induces dermatitis (132), because *Mfge8*-null immature dendritic cells can engulf fragmented apoptotic bodies but not whole dead cells, which are inefficiently degraded. Undigested antigens that accumulate in the immature dendritic cells appear to strongly activate CD8 T cells, causing dermatitis.

In the germinal centers of aged, autoimmune-prone female (NZB × W) F1, BWF1 mice, unengulfed apoptotic cells accumulate and provide self-antigens that cause SLE-type autoimmune disease (133). This is because osteopontin produced from senescence-associated T cells inhibits the MFG-E8-mediated efferocytosis elicited by tingible-body macrophages (133). Treating macrophages with endotoxins downregulates the MFG-E8 expression (134). Accordingly, septic shock is accompanied by a reduced MFG-E8 level in the serum and spleen, causing inefficient efferocytosis (135). This situation appears to increase inflammation, causing death (135). The serum of some SLE patients contains high MFG-E8 levels, or aberrantly spliced, highly glycosylated MFG-E8 (131, 136). A genome-wide association analysis indicated that *MFGE8* is a causative gene of coronary artery disease (137). These results collectively suggest that abnormal MFG-E8-mediated efferocytosis is an etiology of autoimmune diseases.

TIM4

Resident peritoneal macrophages have strong efferocytosis activity, but they do not express MFG-E8 (138). We therefore established a hamster hybridoma library against mouse resident peritoneal macrophages and isolated a monoclonal antibody that inhibits their efferocytosis activity.

Screening a cDNA library from mouse peritoneal macrophages for the antigen recognized by the antibody identified a 343–amino acid type I membrane protein, TIM4 (T cell immunoglobulin- and mucin-domain-containing molecule; also called TIMD4) (138). TIM4's extracellular region consists of an IgG domain and a mucin domain containing *O*-glycosylation and *N*-glycosylation sites. The TIM family has three members in humans and four in mice (TIM1, 2, 3, and 4) (139). TIM4 specifically binds PtdSer via the IgG domain with a K_D in the nanomolar range and confers efferocytosis ability on NIH3T3 cells, indicating that it is the PtdSer receptor for efferocytosis. TIM4 is expressed in various resident macrophages, including resident peritoneal macrophages, Kupffer cells in the liver, $CD169^+$ skin macrophages (140, 141), and $CD4^+$ tingible-body macrophages at Peyer patches in the small intestine (142). *Tim4*-deficient resident peritoneal macrophages or Kupffer cells lose their efferocytosis ability (141, 143), indicating that TIM4 is indispensable for their efferocytosis.

TIM1, also called KIM-1, is specifically expressed in the kidney after injury (144), binds PtdSer with high affinity, and promotes efferocytosis (145). A recent study indicated that TIM1-mediated efferocytosis is important for reducing acute injury to the kidney (146). In contrast to TIM1 and TIM4, TIM2 and TIM3 did not have detectable PtdSer-binding activity in our study (138). However, Nakayama et al. (147) reported that transiently overexpressed TIM3 in 293T cells bound to PtdSer on apoptotic cells. The affinity of TIM3 for PtdSer is very low compared with that of TIM1 or TIM4 (148), and TIM3 may not be involved in efferocytosis (see below).

Protein S, Gas6, and TAM Receptors

PROS, a 676–amino acid (in humans) secreted glycoprotein, is mainly synthesized by hepatocytes and circulates in plasma at approximately 25 mg/L (300 nM) (149). It consists of a Gla domain containing 11 γ-carboxyglutamic acids, 4 epidermal growth factor–like repeats, and 2 globular laminin G–like (LG) domains, and it binds phospholipids via the Gla domain (149). A function of PROS in regulating thrombin generation was revealed in 1980 by Walker, who showed that PROS in the presence of phospholipids enhances the Protein C–mediated inactivation of factors Va and VIIIa (150, 151). Meanwhile, Schneider and coworkers were studying cDNAs whose expression was upregulated in starved cells (152) and found that one of them (GAS6, growth-arrest-specific 6) was homologous to PROS (153).

In 1991, by transfecting chromosomal DNA from human chronic myelogenous leukemia, O'Bryan et al. (154) identified a gene that confers tumorigenicity to NIH3T3 cells and called it *Axl*, from the Greek *anexelekto*, meaning uncontrolled. AXL is a receptor tyrosine kinase containing two IgG and two fibronectin type III domains in its extracellular region, and a tyrosine kinase domain in its cytoplasmic region. Tyrosine kinase receptors with a similar structure were identified by screening a human B lymphoblastoid λgt11 cDNA library with an antiphosphotyrosine antibody (155) and by PCR amplification with degenerate oligonucleotides (156). These genes were designated *Mertk* and *Tyro3*, and with *Axl* they form a subfamily of receptor kinases called TAM (from the first letters of *Tyro3*, *Axl*, and *Mertk*) (157–159). These kinases are expressed differently in different tissues: MERTK in macrophages, retinal pigment epithelium, lung, and small intestine; AXL in LPS-treated macrophages, osteoblasts, ovary, and uterus; and TYRO3 in cerebral cortex, olfactory bulb, and prostate.

In 1995, two groups independently reported that the medium conditioned by some cell lines stimulates AXL's or TYRO3's tyrosine phosphorylation, or TYRO3-dependent cell growth. Factors that bound AXL or TYRO3 were purified and identified as PROS and GAS6 (160, 161). Subsequently, GAS6 was shown to bind MERTK and stimulate its tyrosine-phosphorylation (162), establishing that GAS6 and PROS are ligands for the TAM receptor family. There are

some differences in the affinity of each ligand for different receptors (149, 163, 164). GAS6 binds AXL with much stronger affinity (K_D < 1 nM) than it does MERTK and TYRO3 (K_D 10–30 nM), whereas PROS cannot bind AXL but binds MERTK and TYRO3 with a K_D of 20–50 nM (141).

GAS6 and PROS specifically recognize PtdSer with high affinity in a Ca^{2+}-dependent manner (165, 166) and stimulate efferocytosis, indicating that GAS6 and PROS function as bridging molecules between PtdSer-exposing apoptotic cells and TAM-expressing macrophages. Notably, *Mertk*-deficient mice have defective efferocytosis and develop a lupus-like autoimmune disease with high levels of antinuclear and anti-DNA antibodies (167, 168) that is severely worsened by deleting all three TAM receptors (169). Apoptotic-cell engulfment is accompanied by the secretion of anti-inflammatory or immunosuppressive molecules such as TGF-β, IL-10, and PGE_2, and by a decrease in endotoxin-induced proinflammatory cytokine production (IL-6 and TNF-α). These events were shown to be mediated by TAM receptors (170–172). Thus, *TAM*-deficient mice produce high levels of proinflammatory cytokines, which appears to exacerbate inflammation-associated cancer (173).

Synergistic Effect of the TIM4 and TAM Systems for Efferocytosis

Expressing TIM4 strongly supports efferocytosis in NIH3T3 cells or mouse embryonic fibroblasts (138), but its cytoplasmic region is short, only 42 amino acids, and is dispensable for efferocytosis (174), indicating that TIM4 itself does not transduce the signal for efferocytosis. Many established adherent cell lines such as NIH3T3, 293T, COS, and HeLa express a TAM receptor (164), and deleting the TAM receptor in NIH3T3 cells completely abrogates TIM4's ability to support efferocytosis, indicating that TIM4 requires a TAM receptor for efferocytosis (141). These results indicate that reconstitution experiments of efferocytosis using established fibroblasts and epithelial cell lines should be interpreted carefully. Reconstituting efferocytosis by transforming *TAM*-deficient fibroblasts or B cell lymphoma cells with one of the TAM receptors and TIM4 indicated that TIM4 can recruit apoptotic cells but cannot mediate engulfment. Expressing one of the TAM receptors conferred on fibroblasts a very weak PROS/GAS6-dependent efferocytosis activity, which was strongly enhanced by coexpressing TIM4 (141, 143). These results indicated that apoptotic cell engulfment in TIM4 receptor– and TAM receptor–expressing macrophages occurs in two steps, termed tethering and tickling (175) (**Figure 3**). TIM4 is involved in tethering, and the PROS/GAS6-TAM receptor system in tickling. TIM4 is a type I membrane protein that binds to PtdSer with several-fold higher affinity than does PROS or GAS6, which may be an advantage to support the tethering step. Most macrophages express one of the TAM receptors, and a subgroup of macrophages, including resident peritoneal macrophages, Kupffer cells, and skin macrophages, express TIM4 in addition to the TAM receptor (141). TIM4 is indispensable for efferocytosis by these macrophages, suggesting that these macrophages are specialized to elicit efferocytosis.

Other Proposed PtdSer Receptors for Apoptotic Cells

In addition to the abovementioned molecules, many other membrane molecules have been proposed to recognize PtdSer for apoptotic cell engulfment, including brain-specific angiogenesis inhibitor 1 (BAI1) (176), TIM3 (147, 148), CD300a (177, 178), CD300b (179), CD300f (180), STABILIN 1 and 2 (181, 182), RAGE (receptor for advanced glycosylation end products) (183, 184), and complement C1q (185). However, some of these molecules bind not only PtdSer, but also other phospholipids or proteins, including cardiolipin, phosphatidylinositol, and endotoxins for BAI1 (176, 186); PtdEtn for CD300a (178); TIM1 or TIM4

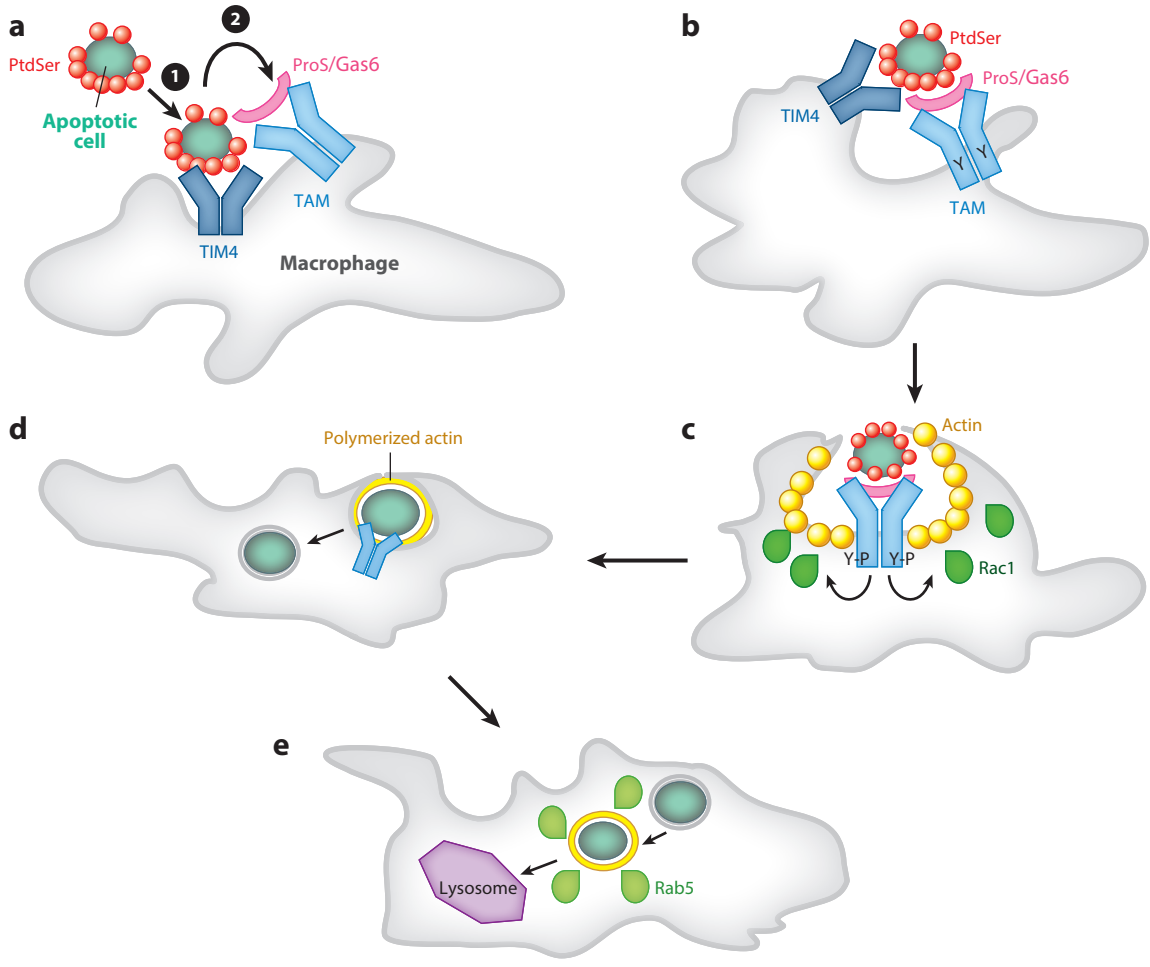

Figure 3

A model for efferocytosis in resident macrophages. (*a*) The entry of apoptotic cells into resident macrophages (resident peritoneal macrophages, Kupffer cells, and skin macrophages) seems to proceed in two steps. (❶) At first, the PtdSer-exposing apoptotic cells are trapped by TIM4, a PtdSer receptor. (❷) Then, the trapped apoptotic cells are passed to TAM receptors (MERTK and Axl in most cells) via ProS and Gas6, which work as an adaptor between the apoptotic cells and TAM receptors. (*b*,*c*) TAM receptors are tyrosine kinase receptors carrying several tyrosine residues (Y) in the cytoplasmic region to be autophosphorylated (Y-P), leading to Rac1 activation and actin polymerization to form actin patches for a phagocytic cup. (*d*) Once the apoptotic cells enter the phagocytes, the phagocytic cups are closed and the actin depolymerized. Phagosomes, vesicles carrying apoptotic cells, travel into lysosomes via repeating the Rab5-induced polymerization and depolymerization of actin. The molecular mechanisms that activate Rac1 and Rab5 for actin polymerization in panels *c* and *e* and what triggers actin depolymerization in panels *d* and *e* have not been well elucidated.

for CD300b (179); ceramide for CD300f (187); DNA, calreticulin, heparin, and pentaxin 3 for C1q (185, 188); hyaluronic acid for STABILIN 2 (189); and HMGB1 for RAGE (190).

Enveloped viruses such as retroviruses, dengue, lentiviruses, and vaccinia expose PtdSer on their surface as apoptotic mimicry (191). MFG-E8 and GAS6 enhance the infection of integrin- and TAM receptor–expressing cells, respectively, by these enveloped viruses (192). TIM1-, TIM4-, or CD300a-expressing cells significantly bound GFP-labeled lentivirus and were more effectively

infected than parental cells, supporting the notion that these molecules function as PtdSer receptors. In contrast, cells expressing BAI1, STABILIN 1, STABILIN 2, TIM3, or RAGEs could not trap the PtdSer-exposing enveloped virus (192). It is not clear why some proteins that recognize PtdSer on apoptotic cells are unable to bind the PtdSer-exposing enveloped virus, although one possibility is that the PtdSer exposed on the virus is positioned or modified differently from that exposed on the apoptotic cell surface.

Other Proposed Molecules in Efferocytosis

In addition to PtdSer, many other molecules have been proposed as eat me signals in apoptotic cells and don't eat me signals in living cells (193). These include annexin I (194) and calreticulin (195) as eat me signals and CD47 (196) and CD31 (197) as don't eat me signals. Annexin I and calreticulin are localized to the cytosol and endoplasmic reticulum, respectively. Weyd et al. (198) and Gardai et al. (195) reported that annexin I and calreticulin are exposed at the early stage of apoptosis similar to the time of PtdSer exposure, while Blume et al. (199) reported that annexin I is externalized on secondary necrotic cells. These molecules can bind PtdSer (200), suggesting that annexin I and calreticulin are exposed together with PtdSer (201), or are released from necrotic cells and then bind to PtdSer on apoptotic cells. Several reports show that annexin I or calreticulin enhances the engulfment of apoptotic cells by phagocytes (194, 195, 202, 203), but another report indicates that annexin I enhances the engulfment of necrotic, but not apoptotic, cells (199). Thus, although we cannot rule out the possibility that annexin I and calreticulin act as eat me signals on apoptotic cells for specific phagocytes, it is probably not a general mechanism.

CD47 is widely expressed in leukocytes and red blood cells. Since CD47-null red blood cells transfused into wild-type mice are quickly cleared by splenic macrophages, Oldenborg et al. (196) proposed that CD47 is a marker of self on red blood cells. Henson et al. (204) further proposed that healthy cells express don't eat me signals, one of which is CD47. CD47 binds to its receptor, SIRPα (signal regulatory protein α), and activates inhibitory phosphatases (SHP-1 and SHP-2) via ITIMs (immunoreceptor tyrosine-based inhibition motifs) in SIRPα, which appears to inhibit macrophage functions such as efferocytosis (205).

Since CD47 expression is strongly upregulated in cancer cells, Weismann's group proposed blocking the CD47-SIRPα axis as an antitumor therapy in which macrophages engulf living tumor cells (206). Calreticulin exposed on cancer cells is thought to be recognized by low-density lipoprotein–related protein expressed by macrophages for engulfment (207), as reported for calreticulin-mediated efferocytosis (195). Notably, anti-CD47 antibodies were reported to have antitumor activity in preclinical studies using a xenotransplantation model with various human tumors (208, 209). However, this was recently challenged by a replication study (210). We and others showed that the CD47 expression is not reduced during apoptosis; rather, it is required for efficient efferocytosis by some macrophage cell lines (211, 212). Potent inhibitors of the CD47-SIRPα axis, including an engineered SIRPα variant that acts as a CD47 antagonist (213) and an anti-CD47 nanobody, did not cause tumor cell engulfment in a syngeneic tumor model in vivo (214). However, when these reagents were combined with an antitumor antibody or with both an antitumor antibody and a T cell checkpoint inhibitor, they caused a massive engulfment of tumor cells by macrophages, showing a synergic antitumor effect. Bian et al. (215) recently reported that a lack of the CD47-SIRPα system activates macrophages and causes a macrophage activation syndrome including hemophilic phagocytosis. They also asserted that calreticulin and LRP1 are an unlikely ligand-receptor pair for the phagocytosis of healthy cells. Thus, how the CD47-SIRPα system discriminates self from nonself is still unclear, but it may not simply act as a don't eat me signal.

APOPTOTIC CELL TRANSPORT INTO THE LYSOSOMES OF MACROPHAGES

When apoptotic cells are recognized by macrophages, various signaling molecules are activated in the macrophages that lead to phagosome formation to encapsulate the apoptotic cells. The phagosomes are transported into lysosomes, where the components of dead cells are degraded into their building units. The formation and cellular trafficking of phagosomes are regulated by small GTPase family members (216–218). Imaging using a FRET sensor for the activated small GTPase showed that apoptotic cells are usually engulfed via lamellipodia, where activated Rac1 is recruited to form a phagocytic cup containing actin patches (219) (**Figure 3**). The phagocytic cup then closes, accompanied by the deactivation of Rac1 and depolymerization of the actin patches. Next, Rab5-mediated actin polymerization mediates the phagosome transport to lysosomes (220). Several other molecules, including DOCK and ELMO for Rac1 activation, and ROCK and mDia for actin depolymerization, are proposed to be involved in this process (221), but much of it remains to be clarified.

FAILURE OR EXAGGERATION OF EFFEROCYTOSIS

Efferocytosis often has a defect in patients with SLE (222). Thus, it was thought that rapid efferocytosis prevents secondary necrotic cells from releasing DAMP, which promotes SLE development (223). In fact, mice deficient in efferocytosis components such as MERTK, MFG-E8, or TIM4 develop an SLE-like phenotype (127, 168, 224). However, this phenotype is very weak, observed in the 129/B6 mixed background but not in the B6 mouse strain (127, 168).

A role of C1q, the first component of the complement system, in efferocytosis has been reported (225). *C1Q* is the strongest susceptibility gene for human SLE (226). Accordingly, its deficiency promotes an SLE-like autoimmunity in mice, but again in a strain-specific manner (227). Several groups recently showed that C1q binds to late apoptotic or secondary necrotic cells, but not to early apoptotic cells (228, 229). Furthermore, unlike the TIM4-TAM-mediated efferocytosis of early apoptotic cells, which proceeds under serum-free conditions (141), C1q-mediated efferocytosis depends on a serum factor (228, 229). These results collectively indicate that efferocytosis has back-up systems (**Figure 4**). In a developmental setting, early-stage apoptotic cells are engulfed via the PtdSer-dependent TIM4-TAM-mediated efferocytosis. When this process does not function efficiently, or the amount of apoptotic cells exceeds the capacity of the macrophages, apoptotic cells undergo secondary necrosis. These cells that undergo secondary necrosis are recognized by the complement system for clearance. In addition, a deficiency of DNase1L3, DNase 1–like serum DNase, causes an SLE-type autoimmune disease (230), indicating that chromatin DNA released from necrotic cells is digested in the circulation, representing another back-up system for preventing autoimmunity induced by dead-cell components. Consistent with this idea, the lupus-prone MRL mouse strain has a missense mutation in the *Dnase1l3* gene (231). If DNA of necrotic cells is digested, engulfment of dead necrotic cells via C1q receptor, Fc receptor, and scavenger receptors such as Marco and MSR1 may not cause inflammation (229, 232, 233). On the other hand, if DNA of necrotic cells is not digested, DNA would promote the formation of a large immune complex, as found with DNA from dying neutrophils (234), and would help activate macrophages and dendritic cells via TLR9 and Fc receptor. This causes the production of inflammatory cytokines including TNF-α and type I interferon to promote autoimmune diseases (235–237) (**Figure 4**). In addition to patients with SLE and glomerulonephritis, those with chronic inflammatory lung diseases (123), such as cystic fibrosis, chronic obstructive pulmonary disease, and asthma, or with atherosclerosis (238) appear to have a defect in efferocytosis. As discussed above

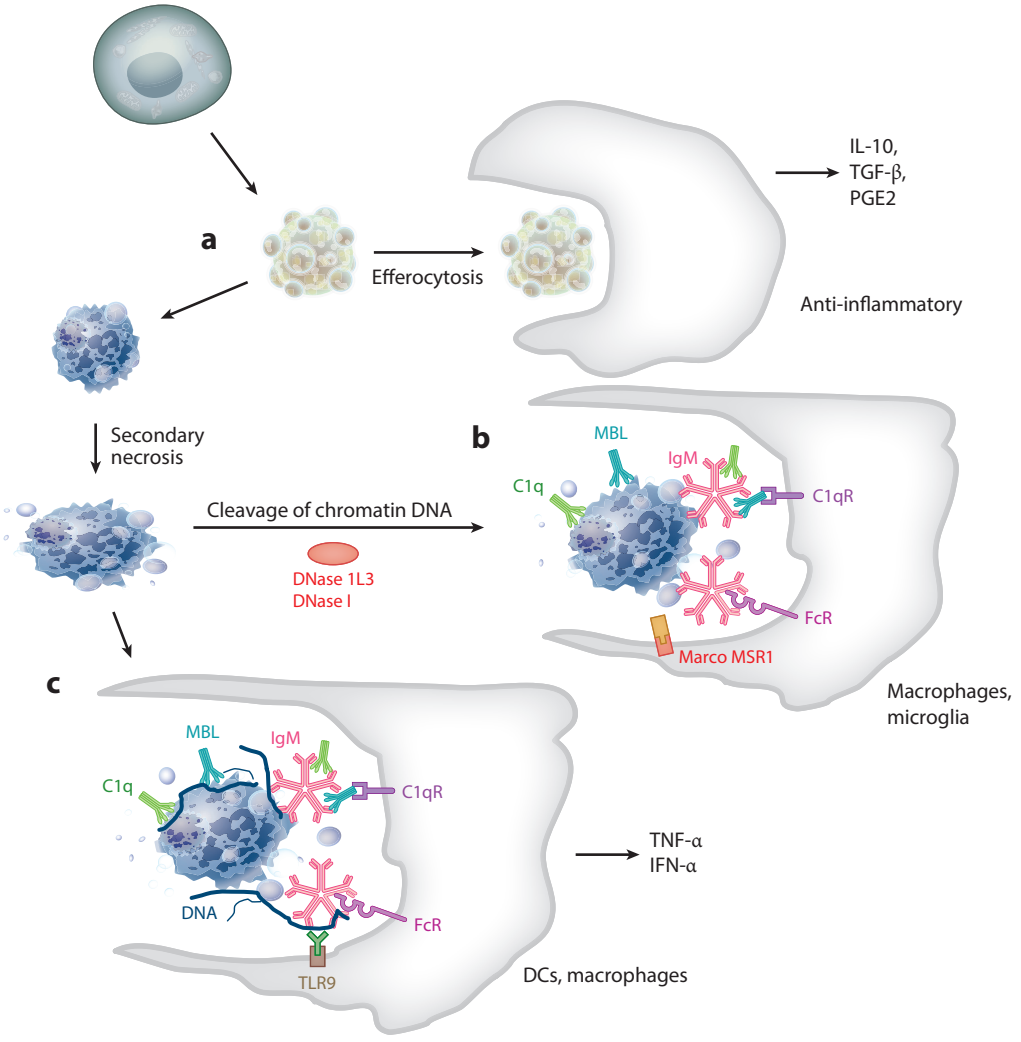

Figure 4

Three systems for clearing components of dead cells. (*a*) After apoptosis, dead cells are engulfed by macrophages via efferocytosis, in which the macrophages engulfing apoptotic cells produce anti-inflammatory molecules such as IL-10, TGF-β, and PGE2. When the efferocytosis does not occur efficiently, the apoptotic cells undergo secondary necrosis. Chromatin DNA of necrotic cells is cleaved by DNase 1 or DNase1L3 (DNase I–like serum DNase). Other remnants of dead cells are recognized and cleared by scavenger receptors (Marco and MSR1) as well as an IgM-, MBL-, or C1q-mediated complement system. This step may not be inflammatory. (*b*) On the other hand, when chromatin DNA of necrotic cells is not degraded, DNA activates macrophages and dendritic cells (DCs) via TLR9 to produce inflammatory cytokines such as TNF-α and IFN-α (*c*). It may also promote the aggregation of immune complex, which can be strongly immunogenic. Figure modified from References 244 and 245.

for SLE-type autoimmune disease, unengulfed apoptotic cells may be responsible for accelerating these diseases via inflammation.

In contrast to failed efferocytosis, which causes inflammation, efferocytosis itself is an anti-inflammatory process that produces immunosuppressive molecules. One of the main tasks of macrophages is to ingest and kill pathogens. However, PGE_2, which is produced during efferocytosis, can suppress the phagocytosis and killing of bacteria by alveolar macrophages in vitro and in

vivo (239). Thus, in addition to its beneficial role in inhibiting inflammation during tissue repair, efferocytosis can decrease antimicrobial responses, which may increase a host's susceptibility to infection.

CONCLUSIONS AND PERSPECTIVES

The number of articles published in 1990 and indexed in PubMed with "apoptosis" or "programmed cell death" in the title or abstract was 135. For 2016, the number was approximately 28,000. Owing to the intensive work of many groups during this quarter century, we have learned how apoptosis is triggered, what kinds of molecules are activated to kill the cells, and how phagocytes recognize the dying apoptotic cells. In addition, we now know that necrosis, once thought to be an accidental death process, is also programmed and have categorized it into pyroptosis and necroptosis. Notably, in 2016, an anticancer drug (venetoclax) developed through apoptosis research was approved by the US Food and Drug Administration for treatment of chronic lymphocytic leukemia (240).

However, the molecular mechanisms of efferocytosis, the engulfment of dying cells, are not yet settled. Many molecules have been proposed as eat me and don't eat me signals, and there is still some confusion surrounding these, just like the early days of apoptosis research. Nevertheless, I am confident that, as in the apoptosis field, the vast efforts of researchers working on efferocytosis will soon clarify these mechanisms. Many human diseases, such as sepsis, autoimmune disease, cystic fibrosis, and asthma, appear to be associated with abnormal efferocytosis. Elucidation of the molecular mechanisms of efferocytosis will contribute to our understanding of these diseases, for which much of the etiology is still unknown.

DISCLOSURE STATEMENT

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