Apoptosis (2019) 24:208–220
https://doi.org/10.1007/s10495-018-01511-x

REVIEW

Methods for monitoring the progression of cell death, cell disassembly and cell clearance

Lanzhou Jiang[1] · **Ivan K. H. Poon**[1]

Published online: 25 January 2019

Abstract

Cell death through apoptosis, necrosis, necroptosis and pyroptosis, as well as the clearance of dead cells are crucial biological processes in the human body. Likewise, disassembly of dying cells during apoptosis to generate cell fragments known as apoptotic bodies may also play important roles in regulating cell clearance and intercellular communication. Recent advances in the field have led to the development of new experimental systems to identify cells at different stages of cell death, measure the levels of apoptotic cell disassembly, and monitor the cell clearance process using a range of in vitro, ex vivo and in vivo models. In this article, we will provide a comprehensive review of the methods for monitoring the progression of cell death, cell disassembly and cell clearance.

Keywords Apoptosis · Necrosis · Pyroptosis · Necroptosis · Apoptotic cell disassembly · Apoptotic bodies · Efferocytosis · Phagocytosis · Cell clearance

Introduction

Over 200 billion cells undergo cell death daily as part of physiological homeostasis and under pathological settings [1]. Cell death can occur through different mechanisms, for example via apoptosis, primary necrosis, necroptosis, pyroptosis and autophagic cell death. Among the different forms of cell death, caspase-dependent apoptosis is thought to account for the majority of homeostatic cellular turnover [2]. Deregulation of this process causes several human disorders including cancer, autoimmune and neurodegenerative diseases [3]. Following cell death, the maintenance of tissue homeostasis depends on the prompt recognition and removal of dying cells by professional and non-professional phagocytes (e.g. macrophages and epithelial cells, respectively). Failure to clear apoptotic cells efficiently will lead to membrane permeabilization, which may promote inflammation through the release of intracellular contents [4]. Prior to cell clearance, certain cell types could also disassemble into smaller membrane-bound fragments (approximately 1–5 μm) known as apoptotic bodies (ApoBDs), a process that may aid cell clearance and intercellular communication [1]. Since cell death, cell disassembly, and cell clearance can contribute to different normal physiological and pathological conditions via distinct mechanisms as described above, it is important to establish suitable methodologies to study each of the cell death processes.

Recently, considerable progress has been made in identifying different stages and molecular regulators of cell death, cell disassembly and cell clearance [4]. A combination of biochemical, flow cytometry and microscopy based approaches are often used to study these processes in vitro and ex vivo. To monitor cell death, cell disassembly and cell clearance in vivo, researchers traditionally rely heavily upon histological and immunohistochemical analysis, and more recently intravital multiphoton microscopy to study these dynamic processes in live animals in real-time. In this review, we will discuss various mammalian experimental systems, in particular state-of-the-art techniques, which can be used to study these cell death related processes.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10495-018-01511-x) contains supplementary material, which is available to authorized users.

✉ Ivan K. H. Poon
i.poon@latrobe.edu.au

Lanzhou Jiang
l.jiang@latrobe.edu.au

[1] Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, VIC 3086, Australia

Methods to monitor cell death

Traditionally, cell death can be differentiated as apoptosis or primary necrosis based on morphological and biochemical differences. Apoptosis is a form of programmed cell death characterized by distinct morphological hallmarks including cell rounding, cell shrinkage, membrane blebbing, nuclear condensation, and cell fragmentation into ApoBDs [5–7]. Other biochemical features of apoptosis include caspase activation, processing of cellular caspase substrates and DNA fragmentation [8–10]. In contrast, primary necrosis is considered a form of accidental cell death resulting from environmental perturbations and does not involve specific signalling pathways [11]. Necrotic cells exhibit morphologies including membrane permeabilization and cytoplasmic swelling. More recently, other forms of cell death have also been identified and described. For example, signalling cascade involving receptor interacting protein kinase 3 (RIPK3) and the pseudokinase mixed lineage kinase domain-like (MLKL), and (in certain settings) receptor interacting protein kinase 1 (RIPK1) have been identified to play a role in the induction of a form of programmed necrosis called necroptosis [12, 13]. Necroptosis has been implicated in mediating many inflammatory diseases including systemic inflammation and progressive atherosclerotic lesions through membrane permeabilization and the release of cellular damage-associated molecular patterns (DAMPs) [14, 15]. Interestingly, necroptosis is also perceived as an immunogenic form of cell death, whereby necroptotic cancer cells can release DAMPs and promote the production of IFN-γ, maturation of dendritic cells (DCs), and cross-priming of $CD8^+$ T cells, consequently inducing a host anti-tumour immune response [16–18]. Moreover, pyroptosis is another regulated form of programmed necrosis that critically depends on the formation of plasma membrane pores by members of the gasdermin protein family; often (but not always) as a consequence of inflammatory caspase activation [13]. Pyroptosis is triggered by perturbations of extracellular or intracellular homeostasis related to innate immunity (e.g. pathogen invasion). The characteristic features of pyroptosis includes the formation of membrane pores generated by gasdermin D (GSDMD) cleavage and the release of proinflammatory cytokines IL-1β and IL-18 [11, 19].

Most experimental assays to date are designed to simply determine whether cells exhibit morphological and biochemical features of apoptosis, and whether cells are membrane permeabilized in order to determine if they undergo necrosis. However, since the discovery of new cell death mechanisms, clearly distinguishing different forms of cell death has become challenging. It should be noted that developing suitable assays to differentiate different types of cell death is critical to determine precisely the regulators of the various forms of cell death and their contribution to disease states. There are a number of recent papers that have reviewed the methods to monitor different forms of cell deaths (especially apoptosis) [13, 20–22]. In the following sections, we provide a brief overview on most commonly used and some latest methods of monitoring cell death in vitro, ex vivo and in vivo.

Monitoring cell death in vitro and ex vivo

Apoptotic and necrotic cells can be characterised in vitro and ex vivo based on their distinct morphological features as aforementioned. The most commonly used techniques for morphology-based apoptosis/necrosis detection include transmission electron microscopic (TEM), scanning electron microscopic (SEM) and time-lapse microscopic analysis [23]. SEM can also be used to distinguish different forms of programmed cell death including apoptosis, necroptosis and pyroptosis based on the plasma membrane changes [18]. Morphologically, necroptosis is characterized by organelle swelling and plasma membrane rupture [18, 24], whereas pyroptosis is marked by GSDMD pore formation and in certain settings the formation of pyroptotic bodies (extracellular vesicles that are 1–5 μm) [25] before the eventual cell lysis. However, morphology-based methods have limitations. In particular, necroptosis, primary necrosis and secondary necrosis following apoptosis eventually result in similar cellular morphology including cytoplasmic swelling, rupture of the plasma membrane and release of intracellular contents [26]. Thus, in order to precisely distinguish different forms of cell death, it is important to monitor biochemical features of cell death in conjunction with morphological analysis.

Molecular biomarkers used in cell death detection include cell surface markers (e.g. phosphatidylserine (PtdSer), pannexin 1 (PANX1) channel activity at the plasma membrane), intracellular markers (e.g. caspase activity, mitochondrial potential), and soluble extracellular markers released by dying cells (e.g. HMGB1 and the enzyme lactate dehydrogenase (LDH)) [23]. Contemporary methods of multiparametric cell death study based on biochemical features can be monitored by fluorescence microscopy, flow cytometry and imaging flow cytometry [22], and these techniques are constantly evolving. For example, traditionally, a combination of annexin A5 (A5, PtdSer binding protein) and either propidium iodide or 7-aminoactinomycin D (PI/7-AAD, membrane impermeable DNA binding dyes) stains are often used to differentiate viable, apoptotic and necrotic cells by flow cytometry [27–29]. Recently, a new flow cytometry-based analytical approach utilizing a combination of A5 and TO-PRO-3 (a DNA binding dye that enters early apoptotic cells through PANX1 channels) stains enables the detection of apoptotic

cells prior to PtdSer exposure, as well as quantifies the levels of ApoBDs and cells at different stages of cell death in a single sample [30]. Although most of the methodologies in cell death research are designed to monitor apoptosis and necrosis, additional approaches have been developed recently to monitor biochemical changes during necroptosis and pyroptosis. Biomarkers that can be used to identify necroptotic cells include the formation of RIP1/RIP3 complex (necrosome), activation of RIPK1 and RIPK3, induction of the RIPK3/MLKL complex, as well as phosphorylation, oligomerization and membrane translocation of MLKL. Techniques such as immunoprecipitation analysis, TEM analysis and immunoblotting can be used to detect these biomarkers [31]. Similarly, biochemical changes during pyroptosis such as the activation of caspase 1, the formation of pyroptosome and processing of GSDMD can also be monitored to detect pyroptosis [13, 32].

Monitoring cell death in vivo

Most of the dead cells and fragments derived from dying cells (e.g. ApoBDs) generated under in vivo conditions are cleared efficiently by professional and non-professional phagocytes [33] before the loss of plasma membrane integrity and release of inflammatory cellular contents [21, 34, 35]. This suggests that regardless of the type of cell death, dead cells are not abundant in most tissues under normal physiological conditions [1, 36]. However, dead cells and debris can often be detected when the level of cell death exceeds the normal situation and beyond the phagocytic capacity of neighbouring phagocytes or when the phagocytes are depleted or dysfunctional [21]. Traditionally, histological and immunohistochemical approaches were heavily used to detect cell death in situ in different organs. The most commonly used method to detect apoptotic cells is terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay. Since positive TUNEL signal may also result from necrotic cells, it is often used with another apoptosis indicator such as active caspase 3 [37]. Another commonly used method is the analysis of soluble extracellular biomarkers released from dying cells (e.g. LDH, hexosaminidase or mitochondrial DNA) in plasma to detect and measure cell death that had occurred in vivo [21]. More recently, the cell death process can be monitored in vivo in real-time at the cellular level in tissues through intravital multiphoton microscopy [38]. For example, by using intravital imaging and transgenic mouse lines expressing a fluorescence resonance energy transfer (FRET)-based caspase 3 activation reporter, morphological changes during apoptosis (e.g. membrane blebbing and ApoBD formation) can be captured in vivo [39, 40].

Methods to study apoptotic cell disassembly

Disassembly of an apoptotic cell into ApoBDs has re-emerged as an area of interest as ApoBDs is considered a major class of extracellular vesicles that could facilitate cell clearance and intercellular communication. Thus, the disassembly of apoptotic cells may also play a key role in diseases such as systemic lupus erythematosus, atherosclerosis and tumourgenesis [1, 41–46]. In recent years, the detection of ApoBDs (differentiation of ApoBDs from cells or other extracellular vesicles) and the functional analysis of ApoBDs have become increasingly important in cell biological, immunological, and extracellular vesicle studies. Techniques that can be used for monitoring ApoBD formation, characterization of ApoBDs, and isolation of ApoBDs are discussed below. It is important to note that the development of suitable methodologies to accurately characterize and isolate ApoBDs is essential for the field of extracellular vesicles as other types of vesicles like microvesicles are often inappropriately described as ApoBDs. It is also worth noting that apoptotic cells can also release vesicles that are smaller than ApoBDs (i.e. apoptotic microvesicles and exosomes-like vesicles) [47], however the methods used to study these subclasses of extracellular vesicles will not be discussed in this review.

Monitoring and characterizing ApoBDs generated under in vitro condition

ApoBDs are membrane-bound extracellular vesicles that are typically 1–5 μm in diameter, larger than microvesicles (50–1000 nm) and exosomes (30–100 nm) [1, 2, 45, 48]. ApoBDs are generated at the later stages of apoptosis, and can be generated from a variety of cell types including T cells, monocytes, endothelial cells and epithelial cells [6, 7, 49–51]. ApoBDs are typically crowded with closely packed organelles and fragments of chromatin [52]. ApoBDs can also carry biomolecules including DNA, microRNA, proteins and lipids, which potentially mediate intercellular communication with healthy cells [50, 51]. Since apoptotic cells can expose PtdSer on the cell surface after caspase activation [53], PtdSer are also distributed on the surface of ApoBDs [30, 54].

Methods for direct visualization of ApoBDs formation in vitro include microscopy-based approaches like histological, TEM and confocal microscopy analysis, as well as flow cytometry-based imaging [7, 49, 55, 56]. In order to monitor the apoptotic cell disassembly process in detail in vitro, such as examining the distribution of cellular contents into ApoBDs or the changes in membrane biomarkers on the surface of ApoBDs, confocal microscopy is generally used for time-lapse and/or fluorescence imaging [6, 49, 51]. Although there are limited studies that quantified the

level of ApoBD formation during apoptosis, ApoBDs can be measured based on their size and/or exposure of PtdSer following an enrichment procedure. For example, methods such as tunable Resistive Pulse Sensing can be used to quantify the amount of large vesicles including ApoBDs in a sample based solely on size [57]. Furthermore, PFA-fixed or ethanol-fixed vesicles that could pass through a 5 µm pore filter can be analyzed by flow cytometry or fluorescence/phase contrast microscopy to quantify the level of cellular fragmentation [7, 58] (Fig. 1a). Similarly, ApoBDs can also be enriched by differential centrifugation and A5-stained/PFA-fixed vesicles are examined by flow cytometry or microscopy analysis [59] (Fig. 1a). However, it should be noted that some of these methodologies have several limitations including the possible undesirable effect of PFA/ethanol-fixation of ApoBDs or the lack of biomarker characterization to confirm the presence of ApoBDs.

In previous studies, the level of ApoBD formation following apoptosis induction can be assessed by flow cytometry based on the size and granularity of cell fragments, and quantified using counting beads [60]. Recently, the level of ApoBDs relative to the amount of apoptotic cells in a sample could also be quantified by flow cytometry using a new analytical strategy and a combination of A5 and TO-PRO-3 stains [30, 49]. Utilizing this flow cytometry-based approach, the apoptotic cell disassembly process under in vitro and ex vivo conditions can be assessed by the ApoBD formation index calculated from the number of ApoBDs divided by the number of A5 positive apoptotic cells [30, 49]. Based on this approach, new analytical methods were also developed for quantitative analysis of ApoBD content such as DNA, RNA and organelles (Fig. 1a) [51]. Notably, DNA and mitochondria are distributed to some but not all ApoBDs and the mechanism of ApoBD formation could affect the distribution of intracellular contents into ApoBDs [51]. Moreover, cell surface molecules such as cell-type specific markers can also be monitored on ApoBDs [51, 61]. It should be noted that a combination of flow cytometry and microscopy imaging analysis should be performed to better define the ApoBD formation process.

Monitoring and characterizing ApoBDs generated under in vivo condition

In addition to monitoring ApoBD formation in vitro, ApoBDs can be detected in vivo in different tissues in the human body as well as in other species including mouse, *Drosophila* and zebrafish [38, 39, 49, 62]. However, since the clearance of dying/dead cells by phagocytes is very rapid under normal physiological settings, the evidence of apoptotic cell disassembly in vivo is still limited [1]. Nevertheless, histology-based methods can be used to study apoptotic cell disassembly and detect ApoBDs in situ in human or rat tissue samples [63, 64] (Fig. 1b). However, in some early studies using hematoxylin and eosin staining, chromatin fragments within apoptotic cells/neighbouring cells or 'tingible bodies' in tingible body macrophages are also described as ApoBDs [65], which does not align with the definition that ApoBDs are extracellular membrane-bound vesicles [6, 61]. Thus, it is important to use appropriate biomarkers to differentiate free apoptotic cells/ApoBDs from the engulfed apoptotic cells/ApoBDs within phagocytes in tissue samples. To achieve this, DNase type I cleavage within apoptotic nuclei, and DNase type II cleavage within apoptotic nuclei in phagolysosome can be distinguished [66] using Apoptag ISOL dual fluorescence apoptosis detection kit [67]. DNase type I cleavage within apoptotic nuclei can also be detected by TUNEL assay [68].

As mentioned earlier, the disassembly of apoptotic cells could be monitored in vivo using intravital multiphoton microscopy [38–40]. For example, by performing two-photon imaging on transgenic mice that express a FRET-based reporter for caspase 3 activity, the process of T cells undergoing apoptosis and fragmentation into small particles were detected [40]. Utilizing a similar approach, cell death dynamics can be monitored in the mice germinal centre where apoptotic B cell fragmentation was detected [39]. Moreover, disassembly and clearance of apoptotic basal epithelial cells in mice hair follicle epithelium tissue can also be monitored by intravital multiphoton microscopy [38]. However, since the tissue is a relatively 'packed' structure, during the apoptosis process the interactions between neighbouring cells and the dying cells could make it difficult to determine if the ApoBD-like structures are 'extracellular vesicles' or particles being 'bitten off' and then transferred within phagocytes.

In order to quantify the level of apoptotic cell disassembly in vivo, flow cytometry analysis similar to methods as discussed above can also be used in some cases. For example, dexamethasone-induced thymocyte apoptosis and ApoBD formation in the mouse thymus can be determined by using a combination of A5, TO-PRO-3, 7-AAD, anti-CD4 and anti-CD8 staining [49] (Fig. 1b). It should be noted that the analytical approach used for determining ApoBD formation generated from in vivo samples is more complex as the sample can contain a number of different cell types [49, 61].

Isolation of ApoBDs

It has been reported that ApoBDs are able to mediate intercellular communication through the transport of various biomolecules [41, 50, 69]. Although the mechanism underpinning the apoptotic cell disassembly process is becoming increasingly clear, the characterization and functional properties of ApoBDs are not well defined [61]. To examine the function of ApoBDs, ApoBDs are typically isolated by

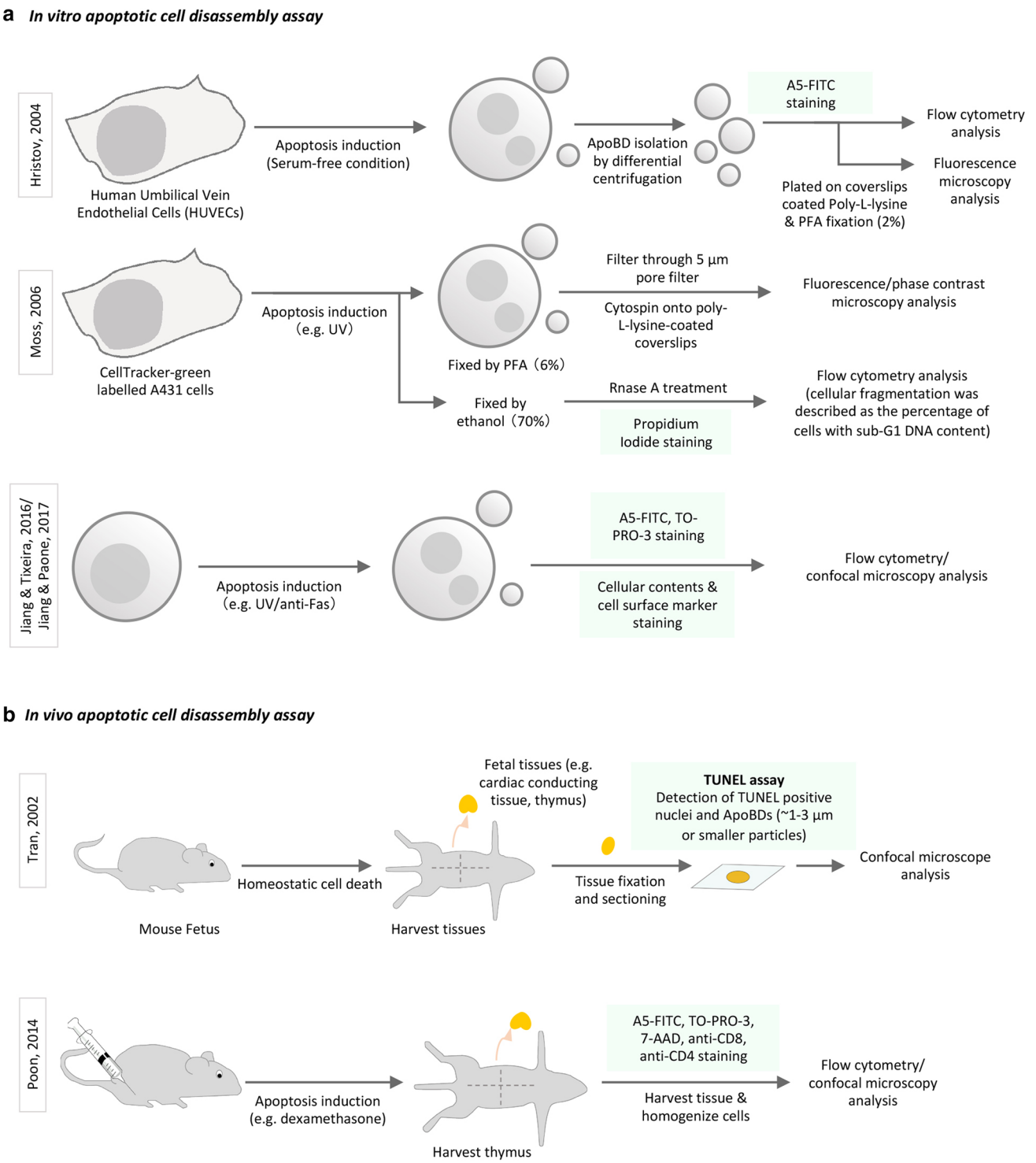


Fig. 1 Analysis of apoptotic cell disassembly. The formation of ApoBDs under in vitro (**a**) or in vivo (**b**) conditions monitored by flow cytometry and/or microscopy-based analysis

differential centrifugation approaches [69, 70]. In previous studies, ApoBD isolation often involves an initial centrifugation at 300–500×*g* to pellet cells, followed by centrifugation of resultant supernatant at 1000–100,000×*g* to pellet ApoBDs [70–72]. It is important to note that other types of extracellular vesicles, in particular microvesicles, may also be present in the isolated ApoBD samples if ApoBDs are pelleted at a high speed [47]. Nevertheless, by using

a differential centrifugation approach (e.g. initial 300×*g* centrifugation followed by 3000×*g* centrifugation), the purity of ApoBDs can be enriched to approximately 84% purity, with a relatively small amount of viable, apoptotic and necrotic cells also present in the sample [61]. By using a low speed centrifugation approach (e.g. centrifugation at 50×*g* for 5 min multiple times to pellet cells and then pellet ApoBDs at 1000×*g* for 6 min), the purity of ApoBDs remains similar [61]. Recently, a fluorescence activated cell sorting (FACS)-based approach was also developed to isolate highly purified ApoBDs [61]. By staining cells and ApoBDs with A5, TO-PRO-3 and cell surface markers, cell-type specific ApoBDs can be isolated from a complex sample (i.e. samples containing different cell types undergoing apoptosis) and purity can reach up to 99% [61]. In addition, ApoBDs can also be isolated by filtration-based alone [7] (Fig. 1a) or a combination of differential centrifugation and filtration-based methodologies [73]. For example, following a 300×*g* centrifugation step to remove cells and cell debris, the resultant supernatant can be filtered through 5 and 1 µm filters to collect vesicles that are between 1 and 5 µm in diameter, followed by ApoBDs pelleted via centrifugation at 2000×*g* for 20 min [73]. There are reports on microfluidics-based technologies for isolating and analysing extracellular vesicles [74], which could be adopted for ApoBD isolation.

Methods to measure dying cell clearance

Rapid removal of dying cells by phagocytes is regulated by various molecular signals and can be divided into a number of sequential steps [36]. First, apoptotic cells can release 'find-me' signals (e.g. nucleotides ATP and UTP, fractalkine, lysophosphatidylcholine and sphingosine 1-phosphate) [75] and recruit phagocytes to the site of cell death. Subsequently, molecules on the surface of the dying cell (e.g. 'eat-me' signal such as PtdSer and calreticulin) [53, 76] and on the surface of phagocytes (e.g. BAI-1, Stabilin-2 and CD91) [2, 77] will interact to initiate efferocytosis [78]. Collectively, exposure of a sufficient amount of 'eat-me' signals and the loss of 'don't eat-me' signals on the surface of apoptotic cells is necessary to trigger their removal by phagocytes [2, 79]. During the efferocytosis process, phagocytes undergo a series of cytoskeletal changes to engulf the dying cell. Finally, cellular debris is digested within the phagolysosomes of phagocytes. Notably, there is also evidence suggesting that apoptotic cells can release factors known as 'keep-out' signals (e.g. lactoferrin) to inhibit the recruitment of inflammatory cells like neutrophils [80].

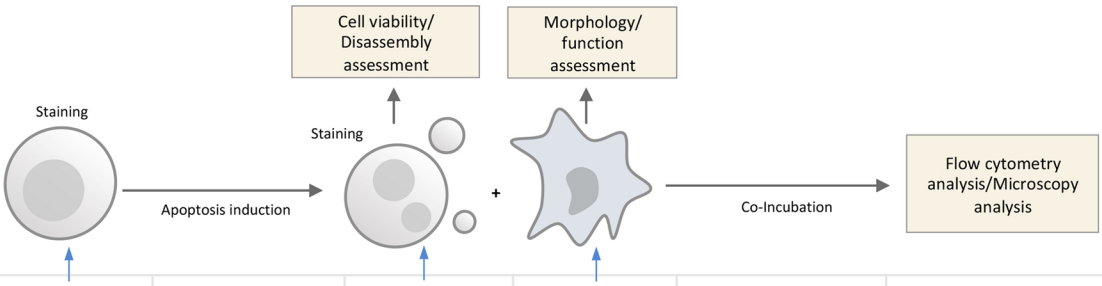
In this review, we will focus on the engulfment step of the overall dying cell clearance processes, known as efferocytosis (certain primary research articles also refer to this process as 'phagocytosis'). Efferocytosis assays are particularly important for defining the role of various molecular signals as described above (e.g. 'find-me' and 'eat-me' signals) in the cell clearance process. A variety of in vitro and in vivo efferocytosis assays are described below (see also Tables 1, 2; Figs. 2, 3). Notably, a typical efferocytosis assay involves the selection of in vitro or in vivo models, suitable target cells, cell death stimuli, type of phagocytes, engulfment environment and method for the quantification of efferocytosis/clearance [81]. Since an efferocytosis assay involves many variables (e.g. target cell:phagocyte ratio, duration of cell death induction and co-culture of target cells with phagocytes), experiments should be designed specifically to address the question of interest. For example, if an in vitro efferocytosis assay is used to examine the mechanism underpinning apoptotic cell uptake, it is important to ensure the majority of target cells are apoptotic and not secondary necrotic within the timeframe of cell death induction and co-culture with phagocytes.

In vitro efferocytosis assays

Target cells used in efferocytosis assays can vary greatly (Supplementary Table 1). For example, in order to test the effect of clusterin (an extracellular chaperone) on apoptotic cell clearance, efferocytosis assays were performed with human cells (freshly isolated neutrophils) and mouse cells (freshly isolated thymocytes) as target cells [82]. In other studies, commonly used target cells include human Jurkat T cells [83, 84], mouse thymocytes [82, 85–88], and human and mouse lung epithelial cells (BEAS-2B/MLE-12) [83, 85]. As for cell death stimuli, commonly used approach to induce target cells to undergo apoptosis include UV irradiation [83, 84], as well as etoposide [83, 85] and dexamethasone treatment (Table 1, Supplementary Table 1) [85–87]. Notably, after apoptosis induction, it is important to confirm the level of target cell apoptosis and necrosis via suitable cell death assays (and apoptotic cell disassembly assays, if needed) as mentioned above to ensure the quality of target cells is validated. The choice of phagocytes can also vary depending on the research question, with the most commonly used phagocytes include mouse bone marrow-derived macrophages (BMDM) [84–86], mouse bone marrow-derived dendritic cells (BMDC) [86] and mouse peritoneal macrophages [85, 88]. Non-professional phagocytes such as MLE-12 lung epithelial cells and fibroblasts are also used in efferocytosis assays [83, 87]. For phagocytes like macrophages, it is recommended to check their morphological characteristics or perform functional assays to determine their ability to engulf (e.g. using synthetic liposome-coated beads as targets) [86] prior to their use in efferocytosis assays.

In order to monitor and measure the efferocytosis process, target cells are often labelled with cell tracer (e.g. cell cytosol stains like CellTracker CFSE [83, 85] or membrane

Table 1 In vitro assays for studying apoptotic cell clearance



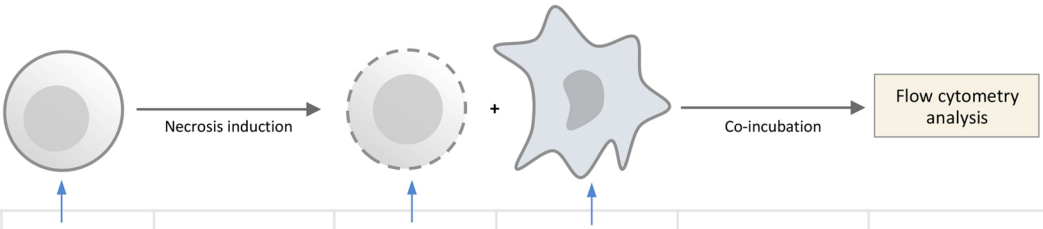
	Target cell	Apoptosis induction	Apoptotic target cell	Phagocyte	Co-incubation condition	Detection & Analysis
Juncadella, 2013	**CFSE/TAMRA labelled Jurkat T cells**	Ultraviolet treatment	**Apoptotic Jurkat T cells**	**MLE-12 cells/** (2.5 μM CFSE stained) **BEAS-2B cells/ primary airway epithelial cells**	Cell ratio*: 10:1 4 h	**Flow cytometry** (1 μM cytochalasin D or purified A5 were used to block efferocytosis)
	CFSE/TAMRA labelled BEAS-2B cells	Etoposide (100 μM) treatment	**Apoptotic BEAS-2B cells**			
Lee, 2016	**CFSE labelled MLE-12 cells/ BEAS-2B cells**	Etoposide (100 μM) treatment	**Apoptotic MLE-12 cells/ BEAS-2B cells** (Assessed by A5/7-AAD)	**Caco-2 cells**	Cell ratio: 10:1 4 h	**Flow cytometry** (1 μM cytochalasin D was added 30 min before co-incubation to inhibit engulfment as a control)
	TAMRA labelled thymocytes	Dexamethasone (50 μM) treatment	**Apoptotic thymocytes**	**BMDM**	Cell ratio: 8:1 45 min	
	CypHer5E labelled thymocytes	Dexamethasone (50 μM) treatment	**Apoptotic thymocytes**	**Peritoneal macrophages**	Cell ratio: 40:1 30 min	
	CFSE labelled MLE-12 cells	Etoposide (100 μM) treatment	**Apoptotic MLE-12 cells**	**HCT-116 cells**	Cell ratio: 10:1-20:1 4 h	
Luo, 2016	**pHrodo Green labelled cells**	Dexamethasone (1 mM) treatment	**Apoptotic thymocytes** (Assessed by A5/ propidium iodide)	**Peritoneal macrophages**	Cell ratio: 5:1 60 min	**Flow cytometry** (anti-F4/80, anti-CD11b staining, doublet discrimination)/ **Microscopy analysis**
Tian, 2016	**Thymocytes**	Dexamethasone (10 μM) treatment (6 h)	**pHrodo labelled apoptotic thymocytes**	**BMDM** (anti-mouse F4/80 staining)	Cell ratio: 3:1 60 min (37 ℃)	**Flow cytometry** (Cells were washed/ suspended in basic buffer pH 8.8 to quench the fluorescence of non-engulfed apoptotic cells before flow cytometry analysis)
				BMDC (anti-mouse CD11c staining)	Cell ratio: 3:1 30, 60 and 120 min (37 ℃)	
			pHrodo/TFL4 labelled apoptotic cells	**BMDC**	Cell ratio: 3:1 60 min (37℃)	**Microscopy analysis** (as per above)
Han, 2016	**Thymocytes (isolated from 4-6-weeks-old mice)**	Dexamethasone	**CypHer5E/ TAMRA labelled apoptotic thymocytes**	**LR73/SVEC-40 cells**	Cell ratio: 10:1 2 h	**Flow cytometry** (Phagocytes are examined: no gross morphological changes occurred due to drug treatment; Target cells were washed with PBS ×3 before analysis)
				BEAS-2B cells	Cell ratio: 5:1 2 h	
				16HBE14o cells	Cell ratio: 20:1 2 h	
Wang, 2017	**Fluorescent/ PKH67/PKH26 labelled Jurkat T cells**	UV (254 nm) lamp for 15 min	**Fluorescent/ PKH67/PKH26 apoptotic Jurkat T cells** (Assessed by A5)	**Peritoneal macrophages**	45 min (or 2-stage efferocytosis#)	**Flow cytometry** (Samples washed with PBS ×3 before analysis)
				BMDM		**Formaldehyde fixation and microscopy analysis**

Table 1 (continued)

*Cell ratio: target cell:phagocyte

#2-stage efferocytosis: PKH67-labelled apoptotic cells were incubated with macrophages for 45 min followed by vigorous rinsing 3 times with PBS. Macrophages were then incubated for another 2 h, followed by addition of PKH27-labelled apoptotic cells. After 45 min, unbound apoptotic cells were removed by rinsing, and then the macrophages were fixed with 4% formaldehyde and imaged on an epifluorescence microscope

Table 2 In vitro assays for necrotic/necroptotic cell clearance studies



	Target cell	Necrosis induction	Necrotic target cell	Phagocyte	Co-incubation condition	Detection & analysis
Poon, 2010	**CFSE labelled Jurkat T cells/ GAG deficient CHO cells**	Hyperthermic conditions (56℃, 30 min)	**Necrotic Jurkat T cells/GAG deficient CHO cells**	**PKH26 labelled THP-1 cells/ $CD14^+$ primary monocytes**	Cell ratio*: 5:1-10:1 60 min (Serum free media, 37℃, 5% CO_2)	**Flow cytometry**
Wang, 2015	**PKH26 labelled HT-29 cells**	Smac mimetic and Z-VAD treatment (48 h)	**Necroptotic BHT-29 cells**	**PKH67 labelled monocyte-derived macrophages**	Cell ratio: 2:1 2 h	**Flow cytometry** (Cell culture medium was also collected for ELISA assay to study the concentration of IL-6 and TNFα)

*Cell ratio: target cell:phagocyte

stains like PKH67 or PKH26 [84]) or a pH sensitive dye (e.g. CypHer5E or pHrodo) to track the internalization of target cells. Notably, levels of efferocytosis in earlier studies were often determined using non-stained target cells and the phagocytic index calculated based on the number of target cells attached on/within phagocytes from image analysis [89, 90]. In most recent studies, the quantification of efferocytosis is often dependent on flow cytometry-based analysis as the target cells are stained with fluorescent dyes [67, 83, 86–88]. However, it is important to note that labelling target cells with CellTracker or PKH dyes has several drawbacks, in particular the difficulties in discriminating apoptotic cells that have been internalized from those that remain bound to the cell surface by flow cytometry [81]. Furthermore, when choosing suitable fluorescent labels for target cells, it is important to avoid fluorophores that fluoresce very weakly at low pH levels as the ingested contents are trafficked to the phagolysosome (pH 4.5–5.5) in phagocytes [91]. Thus, pH sensitive dyes such as pHrodo and CypHer5E are more preferable for efferocytosis assays as their fluorescence increase in acid environment. To complement flow cytometry-based efferocytosis assays, microscopy analysis could also be performed to validate the engulfment of target cells by phagocytes [86]. To clearly distinguish target cells from phagocytes [81], phagocytes can also be stained prior to co-culture with target cells. Similar to apoptotic cell clearance studies as described above, similar principles underpin in vitro efferocytosis assays for necrotic/necroptic cells [92, 93] (Table 2).

In vivo efferocytosis assays

More recently, a number of in vivo efferocytosis assays were established to enable measurement of dying cell clearance under different in vivo conditions. Generally, two different types of strategies are used for these assays. For certain in vivo efferocytosis assays, apoptotic target cells are generated from external origins and delivered to a particular in vivo environment containing phagocytes (e.g. spleen, peritoneal cavity, lung) (Fig. 2). In other types of in vivo efferocytosis assays, target cells are induced to undergo apoptosis in situ and their uptake by phagocytes in a particular tissue (e.g. thymus, colon, spleen) monitored (Fig. 3). In the former approach, target cells are induced to undergo apoptosis in vitro and similar to in vitro efferocytosis assays, these target cells are often labelled with PKH fluorescent dyes or pH sensitive dyes like CypHer5E or pHrodo for internalization analysis [83, 86–88, 94]. Subsequently, apoptotic target cells are administered into mice via intravenous, intraperitoneal or intranasal route depending on the phagocytes of

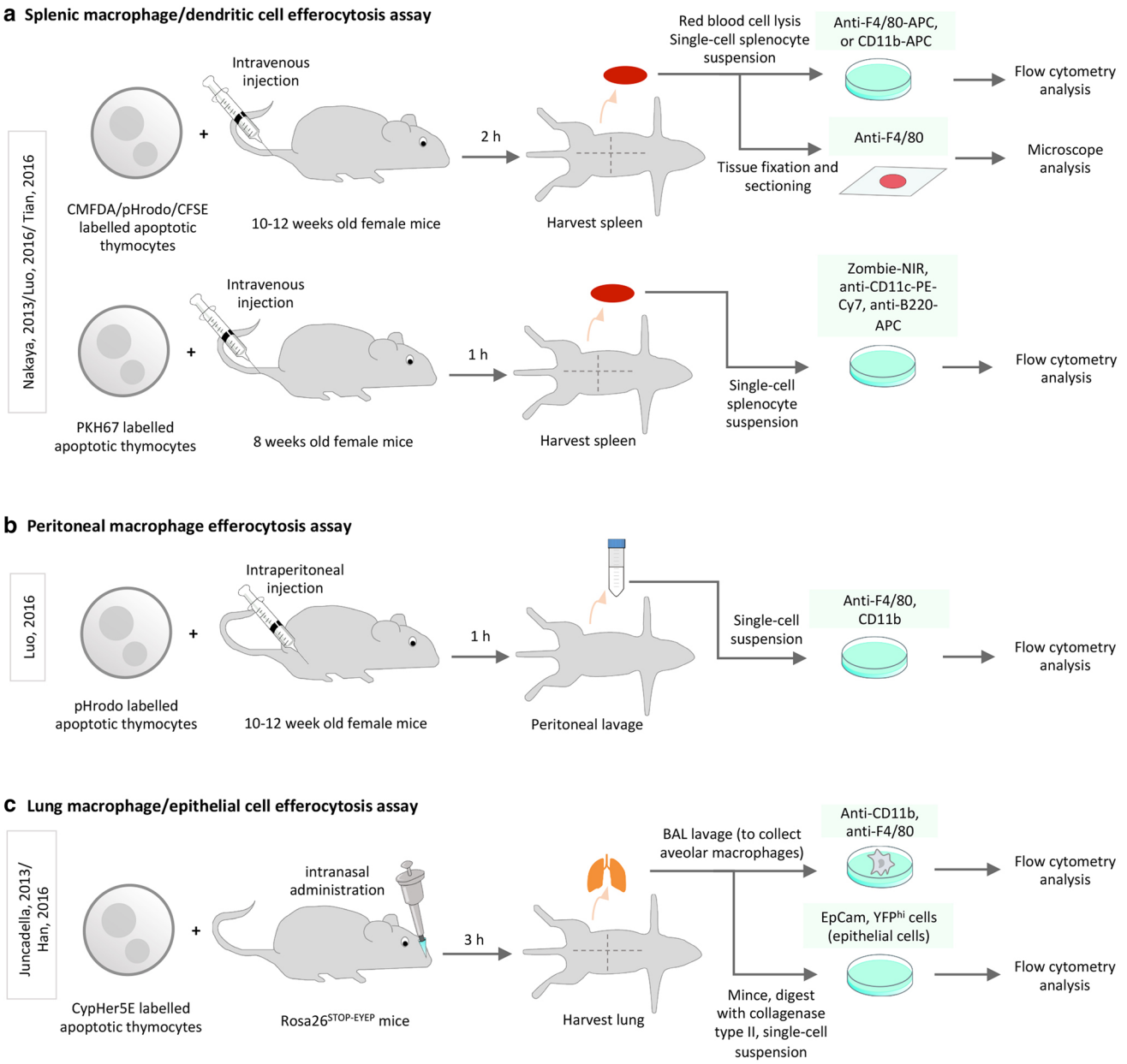


Fig. 2 Apoptotic cell clearance studies using mouse models and apoptotic cells generated from external origins. Phagocytic removal of apoptotic cells delivered into mice via intravenous (**a**), intraperitoneal (**b**), or intranasal (**c**) route monitored by flow cytometry and/or microscopy-based analysis

interest. Typically, after 1–3 h after the administration of apoptotic target cells, phagocytes at the site of interest such as the spleen [86, 94], peritoneal cavity [88] and lung [83, 87] are collected for flow cytometry analysis. In some studies, tissues are fixed and phagocytes are stained for further microscopy analysis [84]. Similar to in vitro efferocytosis assays, it is important to monitor the quality of target cells (e.g. the level of apoptosis and apoptotic cell disassembly) as well as optimize the duration of apoptotic sample administration. Alternatively, when target cells are induced to undergo apoptosis in situ, it is important to identify apoptotic cells accurately. Notably, a number of approaches have been developed to monitor in situ apoptosis and phagocytosis in the thymus [84, 88, 95], colon [85] and spleen [88] (Fig. 3). For example, when monitoring the clearance of apoptotic thymocytes in the thymus, thymocytes are induced to undergo apoptosis by intraperitoneal administration of dexamethasone. Approximately 18–24 h post apoptosis induction, thymus is harvested for the preparation of single-cell suspension or for tissue fixation and sectioning. A5

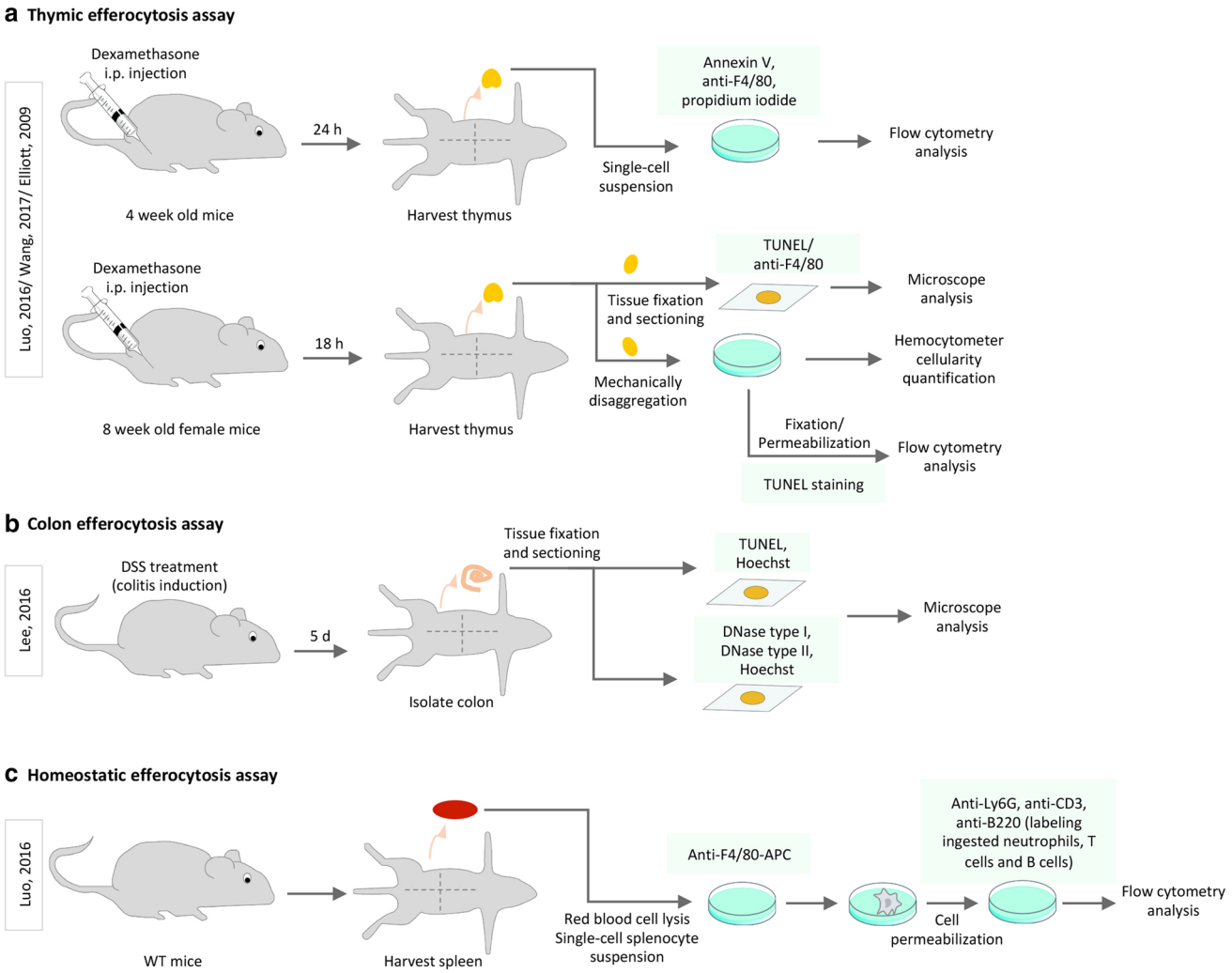


Fig. 3 Apoptotic cell clearance studies using mouse models with apoptosis occurring in situ. Clearance of apoptotic cells generated in situ in the thymus (**a**), colon (**b**), or spleen (**c**) determined by immunohistological and/or flow cytometry-based analysis

or TUNEL staining can then be used to detect apoptotic cells, and macrophages can be identified within the thymus based on F4/80 expression [84, 88, 95]. It is worth noting that phagocytes and apoptotic target cells can be tracked in different species using genetic approaches, which enables the efferocytosis process being monitored in real-time using microscopy-based approaches [38, 81, 96].

Summary

Recent advances in the molecular understanding of different types of programmed cell death has led to the development of new methodologies to differentiate different stages of apoptosis as well as other forms of programmed cell death. Similarly, processes downstream of cell death initiation including apoptotic cell disassembly and cell clearance can also be studied in detail utilizing well established methodologies. Nevertheless, it should be noted that cell death, cell disassembly and cell clearance are interconnecting processes, and preferable to be studied together in physiological relevant settings rather than in isolation. Furthermore, there are still a number of challenges in cell death, cell disassembly and efferocytosis studies, in particular to accurately monitor these processes at the cellular level in vivo. Thus, it is important to continue to develop new approaches to study these cell death processes.

Funding This study was funded by National Health and Medical Research Council (Grant Nos. GNT1125033, GNT1140187) and Australian Research Council (Grant No. DP170103790).

References

1. Atkin-Smith GK, Poon IKH (2016) Disassembly of the dying: mechanisms and functions. Trends Cell Biol. https://doi.org/10.1016/j.tcb.2016.08.011
2. Poon IKH, Lucas CD, Rossi AG, Ravichandran KS (2014) Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol 14:166–180. https://doi.org/10.1038/nri3607
3. Agostini M, Tucci P, Melino G (2011) Cell death pathology: perspective for human diseases. Biochem Biophys Res Commun 414:451–455. https://doi.org/10.1016/j.bbrc.2011.09.081
4. Arandjelovic S, Ravichandran KS (2008) Phagocytosis of apoptotic cells in homeostasis. Nat Immunol 44:280–285. https://doi.org/10.1038/ni.3253
5. Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306. https://doi.org/10.1016/S0074-7696(08)62312-8
6. Atkin-Smith GK, Tixeira R, Paone S et al (2015) A novel mechanism of generating extracellular vesicles during apoptosis via a beads-on-a-string membrane structure. Nat Commun 6:7439. https://doi.org/10.1038/ncomms8439
7. Moss DK, Betin VM, Malesinski SD, Lane JD (2006) A novel role for microtubules in apoptotic chromatin dynamics and cellular fragmentation. J Cell Sci 119:2362–2374. https://doi.org/10.1242/jcs.02959
8. Porter AG, Jänicke RU (1999) Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6:99–104. https://doi.org/10.1038/sj.cdd.4400476
9. Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491–501. https://doi.org/10.1016/S0092-8674(00)81590-1
10. Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89:175–184. https://doi.org/10.1016/S0092-8674(00)80197-X
11. Fink SL, Cookson BT (2005) Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun 73:1907–1916. https://doi.org/10.1128/IAI.73.4.1907-1916.2005
12. Galluzzi L, Kroemer G (2008) Necroptosis: a specialized pathway of programmed necrosis. Cell 135:1161–1163. https://doi.org/10.1016/j.cell.2008.12.004
13. Galluzzi L, Vitale I, Aaronson SA et al (2018) Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ 25:486–541. https://doi.org/10.1038/s41418-017-0012-4
14. Pasparakis M, Vandenabeele P (2015) Necroptosis and its role in inflammation. Nature 517:311–320. https://doi.org/10.1038/nature14191
15. Moriwaki K, Chan FKM (2016) Necroptosis-independent signaling by the RIP kinases in inflammation. Cell Mol Life Sci 73:2325–2334. https://doi.org/10.1007/s00018-016-2203-4
16. Aaes TL, Kaczmarek A, Delvaeye T et al (2016) Vaccination with necroptotic cancer cells induces efficient anti-tumor immunity. Cell Rep 15:274–287. https://doi.org/10.1016/j.celrep.2016.03.037
17. Yatim N, Jusforgues-Saklani H, Orozco S, et al (2015) RIPK1 and NF-κB signaling in dying cells determines cross-priming of $CD8^+$ T cells. Science. https://doi.org/10.1126/science.aad0395
18. Zhang Y, Chen X, Gueydan C, Han J (2017) Plasma membrane changes during programmed cell deaths. Cell Res 28(1):1–13. https://doi.org/10.1038/cr.2017.133
19. Shi J, Gao W, Shao F (2017) Pyroptosis: gasdermin-mediated programmed necrotic cell death. Trends Biochem Sci 42:245–254. https://doi.org/10.1016/j.tibs.2016.10.004
20. Bouchier-Hayes L, Munoz-Pinedo C, Connell S, Green DR (2008) Measuring apoptosis at the single cell level. Methods 44:222–228. https://doi.org/10.1016/j.ymeth.2007.11.007
21. Vanden Berghe T, Grootjans S, Goossens V et al (2013) Determination of apoptotic and necrotic cell death in vitro and in vivo. Methods 61:117–129. https://doi.org/10.1016/j.ymeth.2013.02.011
22. Vorobjev IA, Barteneva NS (2017) Multi-parametric imaging of cell heterogeneity in apoptosis analysis. Methods 112:105–123. https://doi.org/10.1016/j.ymeth.2016.07.003
23. Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P (2008) Apoptosis and necrosis: detection, discrimination and phagocytosis. Methods 44:205–221. https://doi.org/10.1016/j.ymeth.2007.12.001
24. Belizário J, Vieira-Cordeiro L, Enns S (2015) Necroptotic cell death signaling and execution pathway: lessons from knockout mice. Mediators Inflamm. https://doi.org/10.1155/2015/128076
25. Chen X, He W, Hu L et al (2016) Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis. Cell Res 26:1–14. https://doi.org/10.1038/cr.2016.100
26. Vanden Berghe T, Vanlangenakker N, Parthoens E et al (2010) Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell Death Differ 17:922–930. https://doi.org/10.1038/cdd.2009.184
27. Broaddus VC, Yang L, Scavo LM et al (1996) Asbestos induces apoptosis of human and rabbit pleural mesothelial cells via reactive oxygen species. J Clin Invest 98:2050–2059. https://doi.org/10.1172/JCI119010
28. Koopman G, Reutelingsperger CP, Kuijten GA et al (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84:1415–1420
29. Lecoeur H, Ledru E, Prévost MC, Gougeon ML (1997) Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods. J Immunol Methods 209:111–123. https://doi.org/10.1016/S0022-1759(97)00138-5
30. Jiang L, Tixeira R, Caruso S et al (2016) Monitoring the progression of cell death and the disassembly of dying cells by flow cytometry. Nat Protoc 11:655–663. https://doi.org/10.1038/nprot.2016.028
31. He S, Huang S, Shen Z (2016) Biomarkers for the detection of necroptosis. Cell Mol Life Sci 73:2177–2181. https://doi.org/10.1007/s00018-016-2192-3
32. Wallach D, Kang T-B, Dillon CP, Green DR (2016) Programmed necrosis in inflammation: toward identification of the effector molecules. Science 352:aaf2154–aaf2154. https://doi.org/10.1126/science.aaf2154
33. Krysko DV, D'Herde K, Vandenabeele P (2006) Clearance of apoptotic and necrotic cells and its immunological consequences. Apoptosis 11(10):1709–1726. https://doi.org/10.1007/s10495-006-9527-8
34. Kroemer G, Galluzzi L, Vandenabeele P et al (2009) Classification of cell death 2009. Cell Death Differ 16:3–11. https://doi.org/10.1038/cdd.2008.150
35. Dzhagalov IL, Chen KG, Herzmark P, Robey EA (2013) Elimination of self-reactive T cells in the thymus: a timeline for negative selection. PLoS Biol 11(5):e1001566. https://doi.org/10.1371/journal.pbio.1001566
36. Elliott MR, Ravichandran KS (2010) Clearance of apoptotic cells: Implications in health and disease. J Cell Biol 189:1059–1070. https://doi.org/10.1083/jcb.201004096

37. Duan WR, Gamer DS, Williams SD et al (2003) Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. J Pathol. https://doi.org/10.1002/path.1289
38. Mesa KR, Rompolas P, Zito G et al (2015) Niche-induced cell death and epithelial phagocytosis regulate hair follicle stem cell pool. Nature 522:94–97. https://doi.org/10.1038/nature14306
39. Mayer CT, Mayer CT, Gazumyan A et al (2017) The microanatomic segregation of selection by apoptosis in the germinal center. Science 2602:1–14. https://doi.org/10.1126/science.aao2602
40. Garrod KR, Moreau HD, Garcia Z et al (2012) Dissecting T cell contraction in vivo using a genetically encoded reporter of apoptosis. Cell Rep. https://doi.org/10.1016/j.celrep.2012.10.015
41. Bergsmedh A, Szeles A, Henriksson M et al (2001) Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci USA 98:6407–6411. https://doi.org/10.1073/pnas.101129998
42. Ait-Oufella H, Pouresmail V, Simon T et al (2008) Defective mer receptor tyrosine kinase signaling in bone marrow cells promotes apoptotic cell accumulation and accelerates atherosclerosis. Arterioscler Thromb Vasc Biol. https://doi.org/10.1161/ATVBAHA.108.169078
43. Schrijvers DM, De Meyer GRY, Kockx MM et al (2005) Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arterioscler Thromb Vasc Biol. https://doi.org/10.1161/01.ATV.0000166517.18801.a7
44. Seimon T, Tabas I (2009) Mechanisms and consequences of macrophage apoptosis in atherosclerosis. J Lipid Res. https://doi.org/10.1194/jlr.R800032-JLR200
45. György B, Szabó TG, Pásztói M et al (2011) Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci 68:2667–2688. https://doi.org/10.1007/s00018-011-0689-3
46. Baumann I, Kolowos W et al (2002) Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. Arthritis Rheum 46:191–201. https://doi.org/10.1002/art.10027
47. Caruso S, Poon IKH (2018) Apoptotic cell-derived extracellular vesicles: more than just debris. Front Immunol. https://doi.org/10.3389/fimmu.2018.01486
48. Jiang L, Tixeira R, Caruso S et al (2016) [Sup]Monitoring the progression of cell death and the disassembly of dying cells by flow cytometry. Nat Protoc 11:655–663. https://doi.org/10.1038/nprot.2016.028
49. Poon IKH, Chiu Y-H, Armstrong AJ et al (2014) Unexpected link between an antibiotic, pannexin channels and apoptosis. Nature 507:329–334. https://doi.org/10.1038/nature13147
50. Zernecke A, Bidzhekov K, Noels H et al (2009) Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal 2:1–13. https://doi.org/10.1126/scisignal.2000610
51. Jiang L, Paone S, Caruso S et al (2017) Determining the contents and cell origins of apoptotic bodies by flow cytometry. Sci Rep 7:14444. https://doi.org/10.1038/s41598-017-14305-z
52. Saraste A (1999) Morphologic criteria and detection of apoptosis. Herz 24:189–195. https://doi.org/10.1007/BF03044961
53. Nagata S, Suzuki J, Segawa K, Fujii T (2016) Exposure of phosphatidylserine on the cell surface. Cell Death Differ 23:952–961. https://doi.org/10.1038/cdd.2016.7
54. Van Engeland M, Nieland LJW, Ramaekers FCS et al (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31(1):1–9
55. Lane JD, Allan VJ, Woodman PG (2005) Active relocation of chromatin and endoplasmic reticulum into blebs in late apoptotic cells. J Cell Sci 118:4059–4071. https://doi.org/10.1242/jcs.02529
56. Croft DR, Coleman ML, Li S et al (2005) Actin-myosin-based contraction is responsible for apoptotic nuclear disintegration. J Cell Biol 168:245–255. https://doi.org/10.1083/jcb.200409049
57. Osteikoetxea X, Balogh A, Szabó-Taylor K et al (2015) Improved characterization of EV preparations based on protein to lipid ratio and lipid properties. PLoS ONE 10(3):e0121184. https://doi.org/10.1371/journal.pone.0121184
58. Moss DK, Lane JD (2006) Microtubules: forgotten players in the apoptotic execution phase. Trends Cell Biol 16:330–338. https://doi.org/10.1016/j.tcb.2006.05.005
59. Hristov M, Erl W, Linder S, Weber PC (2004) Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. Blood 104:2761. https://doi.org/10.1182/blood-2003-10-3614
60. Wickman GR, Julian L, Mardilovich K et al (2013) Blebs produced by actin-myosin contraction during apoptosis release damage-associated molecular pattern proteins before secondary necrosis occurs. Cell Death Differ 20:1293–1305. https://doi.org/10.1038/cdd.2013.69
61. Atkin-Smith GK, Paone S, Zanker DJ et al (2017) Isolation of cell type-specific apoptotic bodies by fluorescence-activated cell sorting. Sci Rep 7:39846. https://doi.org/10.1038/srep39846
62. van Ham TJ, Mapes J, Kokel D, Peterson RT (2010) Live imaging of apoptotic cells in zebrafish. FASEB J. https://doi.org/10.1096/fj.10-161018
63. Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. J Intern Med 258:479–517. https://doi.org/10.1111/j.1365-2796.2005.01570.x
64. Tran HB, Ohlsson M, Beroukas D, et al (2002) Subcellular redistribution of La/SSB autoantigen during physiologic apoptosis in the fetal mouse heart and conduction system: a clue to the pathogenesis of congenital heart block. Arthritis Rheum 46:202–208. https://doi.org/10.1002/art.10062
65. Elmore SA, Dixon D, Hailey JR et al (2016) Recommendations from the INHAND Apoptosis/Necrosis Working Group. Toxicol Pathol 44:173–188. https://doi.org/10.1177/0192623315625859
66. Evans CJ, Aguilera RJ (2003) DNase II: genes, enzymes and function. Gene 322:1–15. https://doi.org/10.1016/j.gene.2003.08.022
67. Lee CS, Penberthy KK, Wheeler KM et al (2016) Boosting apoptotic cell clearance by colonic epithelial cells attenuates inflammation in vivo. Immunity 44:1–14. https://doi.org/10.1016/j.immuni.2016.02.005
68. Loo DT (2011) In situ detection of apoptosis by the TUNEL assay: an overview of techniques. In: Didenko V (ed) DNA Damage Detection In Situ, Ex Vivo, and In Vivo. Methods in Molecular Biology (Methods and Protocols), vol 682. Humana Press, Totowa. https://doi.org/10.1007/978-1-60327-409-8_1
69. Berda-Haddad Y, Robert S, Salers P et al (2011) Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1. Proc Natl Acad Sci USA. https://doi.org/10.1073/pnas.1116848108
70. Lleo A, Zhang W, Mcdonald WH et al (2014) Shotgun proteomics: identification of unique protein profiles of apoptotic bodies from biliary epithelial cells. Hepatology. https://doi.org/10.1002/hep.27230
71. Berda-Haddad Y, Robert S, Salers P et al (2011) Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1. Proc Natl Acad Sci USA 108:20684–20689. https://doi.org/10.1073/pnas.1116848108
72. Crescitelli R, Lässer C, Szabó TG et al (2013) Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles. https://doi.org/10.3402/jev.v2i0.20677
73. Liu D, Kou X, Chen C et al (2018) Circulating apoptotic bodies maintain mesenchymal stem cell homeostasis and ameliorate

osteopenia via transferring multiple cellular factors. Cell Res. https://doi.org/10.1038/s41422-018-0070-2
74. Guo SC, Tao SC, Dawn H (2018) Microfluidics-based on-a-chip systems for isolating and analysing extracellular vesicles. J Extracell Vesicles 7:1508271. https://doi.org/10.1080/20013078.2018.1508271
75. Ravichandran KS (2010) Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. J Exp Med 207:1807–1817. https://doi.org/10.1084/jem.20101157
76. Panaretakis T, Kepp O, Brockmeier U et al (2009) Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. EMBO J 28:578–590. https://doi.org/10.1038/emboj.2009.1
77. Davies SP, Reynolds GM, Stamataki Z (2018) Clearance of apoptotic cells by tissue epithelia: a putative role for hepatocytes in liver efferocytosis. Front Immunol 9:1–15. https://doi.org/10.3389/fimmu.2018.00044
78. Fadok VA, Voelker DR, Campbell PA et al (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 148:2207–2216
79. Savill J (1997) Recognition and phagocytosis of cells undergoing apoptosis. Br Med Bull 53:491–508. https://doi.org/10.1093/oxfordjournals.bmb.a011626
80. Bournazou I, Pound JD, Duffin R et al (2009) Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. J Clin Invest 119:20–32. https://doi.org/10.1172/JCI36226
81. Platt N, Fineran P (2015) Measuring the phagocytic activity of cells. Methods Cell Biol 126:287–304. https://doi.org/10.1016/bs.mcb.2014.10.025
82. Cunin P, Beauvillain C, Miot C et al (2016) Clusterin facilitates apoptotic cell clearance and prevents apoptotic cell-induced autoimmune responses. Cell Death Dis 7:e2215. https://doi.org/10.1038/cddis.2016.113
83. Juncadella IJ, Kadl A, Sharma AK et al (2013) Apoptotic cell clearance by bronchial epithelial cells critically influences airway inflammation. Nature 493:547–551. https://doi.org/10.1038/nature11714
84. Wang Y, Subramanian M, Yurdagul A et al (2017) Mitochondrial fission promotes the continued clearance of apoptotic cells by macrophages. Cell 171:331–345.e22. https://doi.org/10.1016/j.cell.2017.08.041
85. Lee CS, Penberthy KK, Wheeler KM et al (2016) Boosting apoptotic cell clearance by colonic epithelial cells attenuated inflammation in vivo. Immunity 44:1–14. https://doi.org/10.1016/j.immuni.2016.02.005
86. Tian L, Choi S-C, Lee H-N et al (2016) Enhanced efferocytosis by dendritic cells underlies memory T-cell expansion and susceptibility to autoimmune disease in CD300f-deficient mice. Cell Death Differ. https://doi.org/10.1038/cdd.2015.161
87. Han CZ, Juncadella IJ, Kinchen JM et al (2016) Macrophages redirect phagocytosis by non-professional phagocytes and influence inflammation. Nature. https://doi.org/10.1038/nature20141
88. Luo B, Gan W, Liu Z et al (2016) Erythropoeitin signaling in macrophages promotes dying cell clearance and immune tolerance. Immunity 44:1–16. https://doi.org/10.1016/j.immuni.2016.01.002
89. Trahtemberg U, Mevorach D (2009) Methods used to study apoptotic cell clearance. In: Krysko D.V., Vandenabeele P. (eds) Phagocytosis of Dying Cells: From Molecular Mechanisms to Human Diseases. Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-9293-0_8
90. Hanayama R, Tanaka M, Miwa K et al (2002) Identification of a factor that links apoptotic cells to phagocytes. Nature 417:182–187. https://doi.org/10.1038/417182a
91. Scott CC, Botelho RJ, Grinstein S (2003) Phagosome maturation: a few bugs in the system. J Membr Biol 193:137–152. https://doi.org/10.1007/s00232-002-2008-2
92. Poon IKH, Parish CR, Hulett MD (2010) Histidine-rich glycoprotein functions cooperatively with cell surface heparan sulfate on phagocytes to promote necrotic cell uptake. J Leukoc Biol 88:559–569. https://doi.org/10.1189/jlb.0210087
93. Wang Q, Ju X, Zhou Y, Chen K (2015) Necroptotic cells release find-me signal and are engulfed without proinflammatory cytokine production. Vitr Cell Dev Biol 51:1033–1039. https://doi.org/10.1007/s11626-015-9926-7
94. Nakaya M, Tajima M, Kosako H et al (2013) GRK6 deficiency in mice causes autoimmune disease due to impaired apoptotic cell clearance. Nat Commun 4:1532. https://doi.org/10.1038/ncomms2540
95. Elliott MR, Chekeni FB, Trampont PC et al (2009) Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature 461:282–286. https://doi.org/10.1038/nature08296
96. Erazo T, Coppa A, Pujol A, Bu I (2014) PDR-1/hParkin negatively regulates the phagocytosis of apoptotic cell corpses in *Caenorhabditis elegans*. Cell Death Dis. https://doi.org/10.1038/cddis.2014.57