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Determining the effector response to cell death

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Abstract

Cell death occurs when a pathogen invades a host organism or the organism is subjected to sterile injury. Thus, cell death is often closely associated with the induction of an immune response. Furthermore, cell death can occur as a consequence of the immune response and precedes the tissue renewal and repair responses that are initiated by innate immune cells during resolution of an immune response. Beyond immunity, cell death is required for development, morphogenesis and homeostasis. How can such a ubiquitous event as cell death trigger such a wide range of context-specific effector responses? Dying cells are sensed by innate immune cells using specialized receptors and phagocytosed through a process termed efferocytosis. Here, we outline a general principle whereby signals within the dead cell as well as the environment are integrated by specific efferocytes to define the appropriate effector response.

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Cell death recognition can result in a multitude of distinct effector responses. Here, the authors discuss a framework for determining the specific effector response to cell death that relies on its recognition, contextual environmental signals and identity of the efferocyte.

Keywords

Biological sciences; Immunology; Cell death and immune response; [URI /631/250/1933]; Inflammation; [URI /631/250/256]

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The authors contributed equally to all aspects of the article.

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Introduction

The main function of the immune system is self versus non-self discrimination and defence against foreign invaders while avoiding, as much as possible, self-inflicted damage to host tissues. In these processes, immune cells can both kill and die. On the one hand, $CD8^+$ T cells and natural killer (NK) cells actively kill infected cells or altered host cells, and macrophages can induce cell suicide or apoptosis (BOX 1)[1,2]. On the other hand, immune cells such as activated T cells die during the contraction phase of the immune response[3,4]. Furthermore, immune cells such as macrophages and dendritic cells (DCs) are involved in the recognition and removal of dead cells resulting from injury and infection[5], from homeostatic processes such as the routine death of senescent red blood cells[6], or from embryonic development[7,8]. Thus, cell death is closely associated with the functions of the immune system.

It is clear that the failure of cells to die has serious deleterious immune consequences for organisms. Mice defective in the cell death signalling pathway mediated by FAS (also known as CD95) develop lymphadenopathy, splenomegaly and autoimmune disease[9–11]. In humans, mutations in the genes encoding FAS, caspase 8 or caspase 10 are associated with autoimmune lymphoproliferative syndrome[12–15]. The genetic ablation of BCL-2-interacting mediator (BIM), a pro-apoptotic protein, results in excessive numbers of lymphoid and myeloid cells and autoimmune disease[16]. What is less clear is the relationship between cell death, its recognition and disposal and the specificity of the subsequent effector responses. For example, cells such as neutrophils die by apoptosis during tissue repair responses after injury[17]. Normally, apoptotic cell death is associated with tissue repair and not inflammation[18]. However, apoptotic cell death during sterile tissue damage or infection can induce inflammation, either alone or accompanied by the activation of the adaptive immune response[19,20]. Thus, apoptosis can be associated with both non-inflammatory and inflammatory effector responses. The homeostatic cell death of red blood cells can trigger their clearance by splenic red pulp macrophages, but this is associated with neither tissue repair nor inflammation, albeit the neighbouring white pulp of the spleen is an epicentre for adaptive immunity and the marginal zone is involved in both innate and adaptive immunity[21,22].

The array of effector responses to cell death is large, including many forms of immune defence, distinct tissue maintenance functions and developmental functions such as digit formation and neuronal circuit development. How can the complex and multidimensional information that results in such an array of effector responses be encoded in cell death? Can we unravel this complex code to predict the effector responses to cell death that shape our biology? The concept that different types of cell death result in specific effector responses is not new but here, we discuss the possibility that the cell type that engulfs the dead cell during its removal, known as the efferocyte, integrates signals encoded by both the dead cell and the environment of cell death to execute a specific effector response (FIG. 1). The efferocyte is typically a professional phagocyte of the immune system such as a macrophage or DC, although in some cases, the efferocyte may be a non-professional phagocyte such as a fibroblast or endothelial cell. Notwithstanding, we propose that the efferocyte holds the secret to the specificity of the effector response when a cell dies.

The reductionist approach

In the simplest scenario, there could be a one-to-one correlation of cell death with effector function, such that the biological consequences of cell death are carried out by the act of death itself. In theory, a specific form of programmed cell death driven by a distinct molecular mechanism could encode a unique effector function. For example, cell death during development or homeostasis is primarily apoptotic[23–27] and is not associated with antimicrobial activity or inflammation. By contrast, death by NETosis (BOX 1) of neutrophils during an infection ensures the ensnarement of invading bacteria and other pathogens within DNA nets[28]. Cell death during *Salmonella* infection can occur by pyroptosis (BOX 1) and is associated with significant inflammation[29]. A corollary, then, would be that the degree of inflammation associated with a specific form of programmed cell death correlates with physiological versus pathological outcomes of cell death (FIG. 1).

However, the number of types of cell death is fewer than the number of unique effector responses such that a single type of cell death is not restricted to a single effector function, which argues against a one-to-one correlation between cell death and effector function. Apoptosis has been categorized as generally anti-inflammatory and pyroptosis has been deemed inflammatory. However, death by apoptosis or pyroptosis can elicit multiple effector responses. Apoptotic death during development[30,31] and during homeostatic cellular turnover in adults[32,33] is not associated with inflammation, whereas apoptotic death during infection or in sterile tissue damage does induce inflammation[19,20]. Apoptotic death in the context of infection can activate the adaptive immune response, whereas in the context of sterile injury it could be limited to inflammation[19,20]. It should also be noted that components of the molecular machinery of apoptosis, such as caspase 3, have recently been implicated in inflammatory cell death through the activation of gasdermin E[34]. Thus, apoptosis is associated with both non-inflammatory and inflammatory forms of cell death and with several, distinct effector responses[35]. A similar relationship has recently been described for pyroptosis. This form of cell death is mostly associated with infection and the production of pro-inflammatory mediators such as IL-1 and IL-18, but a recent study reported the requirement of pyroptosis in neurodevelopment[36]. Pyroptosis triggered in a sterile environment through the sensing of neuronal DNA damage resulted in the elimination of neurons in a gasdermin-D-dependent manner[36]. Of note, IL-1 or IL-18 signalling were not required for this developmental function of pyroptosis[36]. Therefore, a reductionist approach of dissecting cell death to its molecular mechanisms does not seem to hold all of the clues to the effector response.

Integrating cell death and clearance

To understand the consequences of cell death for an organism, here we posit considering cell death together with the cell type responsible for its disposal. Cell death is closely associated with its recognition and clearance, and the effector function associated with cell death is manifested during its disposal. For example, resolution of pulmonary inflammation is associated with the engulfment and clearance of apoptotic neutrophils by macrophages[17]. Similarly, apoptotic cells introduced intravenously while grafting bone marrow in mice are removed by host macrophages, which facilitates engraftment of the donor bone marrow[37].

The engulfment of dead cells by phagocytes (known as efferocytosis) is frequently observed in homeostasis[32]. Phosphatidylserine (PtdSer) is displayed on the outer membrane leaflet of apoptotic cells and is recognized by receptors that initiate efferocytosis[38,39]. Sequestering PtdSer by the microinjection of a PtdSer-binding protein in seminiferous tubules of mice resulted in the accumulation of apoptotic cells and decreased number of spermatogenic cells and epididymal sperms[40]. The failure of efferocytosis during development can be associated with morphogenetic defects. In *Drosophila*, RNA interference of *croquemort* (which encodes a hemocyte and macrophage receptor for apoptotic cells) resulted in central nervous system defects[41]. The inhibition of macrophage function during amphibian or mammalian development also leads to morphogenetic and developmental defects[42,43]. A clear example of the integration of cell death with its clearance is seen in *Caenorhabditis elegans.* The combination of a mutation that renders cells partially defective in undergoing cell death (*ced-3* partial loss of function) with mutations in engulfment genes (*ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10* and *ced-12*) leads to the survival of cells that are otherwise destined to die and the presence of supernumerary cells in the organism[44,45].

Thus, in contrast to a reductionist view that cell death encodes information specifying the effector programme to be carried out by a passive responder, we propose that cell death requires the participation of an active intermediary to execute the encoded tasks and that the effector response can be understood in terms of a composite of the nature of cell death, the cellular responders to cell death and the context of cell death (FIG. 1). In this regard, we posit three factors that determine the effector response, which can be described by the formulae given in BOX 2. First, is the identity of the cell that recognizes and engulfs the dying cell. Second, is the information content of the dying cell beyond the molecular mechanisms responsible for cell death, which may involve shared molecules that are displayed or released by dying cells as well as unique signals present in the specific dying cell. Third, is the contextual framework of cell death, which involves the integration of signals from surrounding living tissue as well as from the dead cell. Every factor represents a set of variables, but a sequence of constraints at each level limits the outcomes. For example, approximately fifteen distinct molecular mechanisms of programmed cell death have been described[46], some of which are exclusively triggered in specific environments (for example, pyroptosis producing IL-1 and IL-18 in the setting of infection and inflammation)[47]. Thus, the environment can constrain the information encoded in cell death. Constraints can also occur at the level of the efferocyte that engulfs the dead cell. The metabolic and/or transcriptional capabilities of distinct efferocytes, such as infiltrating monocyte-derived macrophages, tissue-resident macrophages or epithelial cells, restrict their tissue distribution and effector responses. Finally, we posit that the whole may be different than the sum of its parts. The nature of the efferocyte not only constrains the possible responses but also is the operational system within which integration can occur. Thus, the effector response is not merely selected as one of several possible outcomes specified by the dying cell or by the environment alone but also can be different from these outcomes. In summary, collective information provided by the type of cell death, environment and effector cell, together with a multifactorial series of constraints, is integrated to specify a single output (BOX 2). Below, we develop this integrated theory to provide a rough guide for the study of the physiological and pathological effector responses to cell death.

The role of the efferocyte

The efferocyte has a crucial role in determining the effector response to cell death (FIG. 2). Living neighbouring cells carry out specific effector functions that are necessary for the development or survival of the tissue and/or organism following a cell death event. In *C. elegans*, dead cells are removed by their sessile neighbours[48]. However, in most phyla, disposal is mainly carried out by professional phagocytes known as macrophages. DCs are also capable of efferocytosis of apoptotic cells and infected apoptotic cells[19]. That said, several other cell types, including epithelial cells, fibroblasts and endothelial cells, are also known to engulf dead cells[49–52]. In fact, it has been proposed that most cells can function as efferocytes in some circumstances[53]. Here, we postulate that the identity of the efferocyte, at least partially, determines a composite response to cell death.

One efferocyte, one response.

It is possible that a primary type of efferocyte is designated for a specific cell type that is programmed to die (FIG. 2a). The prevalence of a designated primary efferocyte at various points in development or at different locations in tissues can respond to the cell death that is destined to occur in time and space. In turn, the identity of the efferocyte may define the effector response. In the brain, microglia are the professional efferocytes, but neuronal progenitors, glial precursors and astrocytes can also efferocytose neuronal and axonal debris[54–60]. Microglia are present in the developing brain from embryonic day 9 (E9)–E10 in mice[61]. One study estimated that 70% and 50% of cortical cells, primarily progenitor cells, die at E14 and E18, respectively, during mouse brain development[62]. Thus, the microglia are poised to remove cells that die during early brain development. By contrast, cortical astrocytes begin to emerge only around E16–E18, with the bulk of them emerging around postnatal day 7 (P7)[61]. Co-incidentally, a second, larger wave of programmed cell death of specific post-mitotic neuronal populations is observed in the cortex in the first to second week after birth[63]. Therefore, astrocytes as well as microglia are available to clear these dying post-mitotic cells. The assignment of a specialized efferocyte to a specific population of dying cells during development may pair a specific effector function to the death of those cells. In the case of neural development, the specific effector function is unknown. However, one example of a dedicated pairing of type of efferocyte with dying cell type, leading to a specialized effector function, is that of splenic red pulp macrophages, which engulf dying red blood cells and metabolize the iron that they contain[64].

Multiple efferocytes, different responses.

Alternatively, different efferocytes may be involved in the disposal of a particular dying cell type, resulting in distinct effector responses (FIG. 2b). For example, both alveolar macrophages, which are professional phagocytes, and bronchial epithelial cells engulf apoptotic cells in lung airways[49]. Although the consequences of efferocytosis by these cell types remain to be directly described, it was reported that alveolar macrophages release insulin-like growth factor 1 (IGF1) while phagocytosing apoptotic cells, which in turn reduces efferocytosis by bronchial epithelial cells[50]. Thus, efferocytosis by non-professional phagocytes can be tuned by professional phagocytes. Presumably, this redirects efferocytosis to macrophages and perhaps instructs the physiologically appropriate response. Although

bronchial epithelial cells, similarly to macrophages, can suppress inflammation in an IL-10-dependent manner following efferocytosis[49], the absence of IGF1 receptor on bronchial epithelial cells resulted in exacerbated lung inflammation after allergen exposure[50]. Another example of multiple efferocytes clearing a particular dead cell type occurs in the context of the intestine. Apoptotic intestinal epithelial cells are engulfed by a subset of DCs and two distinct subsets of macrophages, each of which has a unique gene expression signature that suggests a specialized effector function[65]. The two macrophage subsets upregulate genes involved in phagosome maturation, lipid metabolism and branched-chain amino acid catabolism after efferocytosis[65]. By contrast, DCs upregulate genes involved in regulatory T (T_{reg}) cell recruitment and proliferation, and CC-chemokine receptor 7 (CCR7)[65]. Similarly, both fibroblasts (human dermal, lung or kidney) and macrophages can engulf apoptotic neutrophils in vitro[51,66], although the resulting effector functions are unknown. Furthermore, the effector response following phagocytosis by differentially polarized macrophages can be expected to be dissimilar if one considers their polarization states as being at opposite ends of a gene expression spectrum.

One efferocyte, different responses.

Although different cell types can partake in efferocytosis and thereby contribute to the diversity in effector responses, even the same efferocyte can mediate different responses (FIG. 2c). For example, macrophages can support the restoration of homeostasis and regeneration, as well as scarring. During hepatocyte regeneration following chronic liver injury, the efferocytosis of hepatocyte debris by macrophages and the resultant release of WNT3A promote specification of hepatic progenitor cells into hepatocytes[67]. By contrast, macrophage depletion during carbon tetrachloride-induced liver fibrosis results in reduced scar formation[68]. Similarly, following punch biopsy injuries in the skin of mice, early depletion of macrophages (in the inflammatory phase) results in delayed wound healing but decreases the formation of scar tissue[69].

How can the same efferocyte respond differently? The diversity of responses from identical efferocytes may result from the large number of efferocytosis receptors and downstream pathways. In *C. elegans*, two parallel pathways of efferocytosis have been described[70]. In mammals, the best known efferocytosis receptors are PtdSer sensors. PtdSer is recognized directly by several receptors including BAI1 (also known as ADGRB1), TIMD4, CD300LF, STAB1 and STAB2[38]. Furthermore, exposed PtdSer can be tethered indirectly to TAM (TYRO3, AXL and MERTK) receptor tyrosine kinases or to MEGF10 through the soluble ligands GAS6 and PROS1 or C1q, respectively[38].

The interpretation that different receptors may endow an efferocyte with functional specialization is consistent with tissue-resident macrophage populations having heterogeneous expression of phagocytic receptors[71]. Perhaps more surprising is that the same cell type can express multiple efferocytosis receptors at the same time. For example at P21 in mice, liver-resident macrophages express *Timd4* and *Mertk*, whereas kidney-resident macrophages express high levels of *Timd4* and *Stab1*[72]. Human dermal fibroblasts use vitronectin receptor or a mannose/fucose-specific lectin for efferocytosis[51]. Although tissue-resident macrophages and fibroblasts are unlikely to be a homogenous species, analyses of

single-cell RNA-sequencing data indicate that a single efferocyte can indeed express multiple efferocytosis receptors. For example, AXL and TIMD4 are both expressed by individual mouse liver macrophages (see the VirtualCytometry website).

Assuming that the expression of multiple receptors by a single cell is not simply built-in redundancy, this observation suggests that the engagement of a specific receptor or pathway may signal a distinct effector response. For example, two different efferocytosis pathways have been described for the removal of Corazonin-expressing neurons in the ventral nerve cord (vCrz$^+$ cells) of *Drosophila* larvae. Whereas Draper was required for the elimination of vCrz$^+$ cell bodies, the Crk–Mbc–dCed-12 pathway, but not Draper, was required for the removal of vCrz$^+$ neurites. Interestingly, *draper* mutants, but not mutants of the Crk–Mbc–dCed-12 axis, had preserved and morphologically intact vCrz$^+$ neurons[73]. These results suggest that Draper-dependent phagocytosis may promote neuronal apoptosis[73], whereas the Crk–Mbc–dCed-12 pathway may be involved in circuit remodelling. Another example is provided by MERTK, which is expressed by retinal pigmented epithelial cells in the eye and by Sertoli and Leydig cells in the testes[74,75]. *Mertk*$^{-/-}$ mice are characterized by retinal degeneration, and male *Mertk*$^{-/-}$*Axl*$^{-/-}$ mice or *Mertk*$^{-/-}$*Tyro3*$^{-/-}$ mice have reduced testis weight, sperm count and fertility[75,76]. The expression of BAI1 in *Mertk*$^{-/-}$ mice rescues the clearance of apoptotic germ cells in the testes but fails to prevent or reduce photoreceptor degeneration[77], which points to redundancies, as well as functional specializations, between these two efferocytosis receptors. Both BAI1 and MERTK can participate in the efferocytosis of apoptotic germ cells, but only MERTK and not BAI1 can function in the efferocytosis of photoreceptors. Although in this example two different cell types are involved, theoretically the phagocytic removal of a dead cell by two distinct receptors within a single cell could be coupled to the production of discrete sets of downstream factors.

In summary, there are at least three ways in which cell death can be paired with particular efferocytes to specify the appropriate effector response. There could be a dedicated primary efferocyte assigned to respond to programmed cell death occurring at a precise time and place, which would allow for the evolution of specialized effector functions. A downside of this system could be the lack of an alternative means of disposal such that functional deficits in the efferocyte are likely to be fatal or associated with severe disease. Alternatively, different efferocytes might be available to dispose of the dead cell, but they are organized hierarchically or must crosstalk to establish a dynamic hierarchy. Each efferocyte has a fixed effector function, thus the hierarchy determines the effector function. Finally, a single efferocyte could be capable of distinct effector responses, perhaps resulting from the choice of receptor it uses to sense the dead cell. These latter two scenarios allow for specialization with a degree of failsafe. Although the failsafe measures may not be complete substitutes of the required function, some degree of redundancy or compensation may be possible, which would reduce the mortality or severity of the diseases associated with defects in a particular type of efferocytosis.

Related links
VirtualCytometry: https://www.grnpedia.org/cytometry

The role of the dying cell

The second factor in our theory is the information contained in the dying cell (FIG. 3). For an in-depth review of the different types of cell death, which we do not intend to cover here, the reader is referred to REF.[46]. If the identity of the efferocyte is some kind of an operational environment for decoding information, the source code is represented by the dying or dead cell.

Type of cell death.

It has been previously described that the molecular machineries of specific forms of cell death can specify the appropriate effector response, such as in the case of immunogenic versus tolerogenic responses[78,79]. The specific molecular modality of cell death by NETosis can trap and kill bacteria[28]. Pyroptotic death of macrophages can generate structures known as pore-induced intracellular traps that trap and damage live bacteria[80]. Moreover, these dead macrophages together with the trapped bacteria provide specific signals for complement and scavenger receptor activation, and the recruitment of macrophages and neutrophils for effective bacterial killing[80]. A specific functional relationship between pyroptosis and anti-bacterial effector responses can also be inferred from the elaborate defence mechanisms in bacteria that are dedicated to avoiding inflammasome activation and hence pyroptosis[81,82]. IL-18, which is released during pyroptosis, can also induce IFNγ and T helper 1 (T_H1) cell responses[83], and the other pyroptosis-associated cytokine IL-1β can promote T_H17 cell differentiation[84,85]. Admittedly, IL-1β and IL-18 are not synonymous with inflammasome activation and inflammasome activation can occur without pyroptosis[86], but this form of cell death may contain some information to generate a specific anti-bacterial effector response. However, it is important to note that pyroptosis is not exclusive to bacterial infections and can also occur following viral infections, Alzheimer disease or liver disease and even during neuronal development[36,87–89].

Another form of programmed cell death, necroptosis (BOX 1), is associated with the release of danger signals in the form of damage-associated molecular patterns (DAMPs) such as ATP and high-mobility group box 1 (HMGB1). It is likely that cellular sensors of PtdSer functioning together with cytokine receptors or DAMP sensors translate pyroptotic or necroptotic cell death into a specific effector response or lack of response. HMGB1 has been reported to inhibit efferocytosis by competing with PtdSer for receptor binding, as well as by sequestering $\alpha_v\beta_3$ integrins on the surface of phagocytes[90,91]. Finally, lactic acidosis associated with acute tumour lysis syndrome, which results from lactate production following the loss of mitochondrial membrane potential during apoptosis[92], has been shown to directly affect macrophage polarization and effector function[93].

Therefore, specific forms of cell death can contain information for specifying the effector response. Interestingly, infection can change the mode of cell death and, thus, the information coded therein. Intracellular community-associated methicillin-resistant *Staphylococcus aureus* suppresses the apoptosis of neutrophils and inhibits their efferocytosis by macrophages. This redirects infected neutrophils to undergo programmed necrosis[94] (BOX 1). Whether this change in the type of cell death directly affects the effector response is not known, but macrophage effector responses after *S. aureus* infection are

reprogrammed to produce more IL-8 (also known as CXCL8) and less tumour necrosis factor (TNF) and IL-1β[94].

Plasticity between cell death modalities.

The programmed cell death of a single cell does not occur through a predetermined route and plasticity between different modalities of cell death has recently been uncovered[95,96]. For example, TNF signalling can induce apoptosis or necroptosis through caspase 8 or receptor-interacting serine/threonine-protein kinase 3 (RIPK3), respectively[97]. Interestingly, although *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice, which have defective necroptosis, develop normally[98–100], *Casp8* deletion, which prevents apoptosis, results in embryonic lethality owing to excessive necroptosis[101–103]. Thus, one form of cell death (apoptosis) keeps at bay the other (necroptosis) and its associated deleterious consequences. Implicit in this phenomenon is the idea that the modality of cell death codes for specificity of the effector response. Whether or how the efferocytes involved interpret embryonic apoptosis versus necroptosis to execute the appropriate effector response remains unknown.

Number of dead cells.

The number of cells that die at a time could also contain quantitative information that is coordinated by efferocytes to mediate the appropriate response. For example, macrophages can organize a predictive response to the extent of injury; plucking a certain density of hair from mouse skin can stimulate the regrowth of plucked as well as unplucked hairs, but plucking hairs below this threshold density does not result in hair regeneration[104]. Even if the same number of hairs were plucked, the area from which they were plucked was crucial. It is not known whether the role of macrophages in sensing dead cells plays a role in this process, but it is likely that a combination of cytokines, chemokines and macrophages are involved[104]. Macrophages can phagocytose cargoes sequentially. Apoptotic cells can be engulfed by macrophages through LC3-associated phagocytosis **[G]** (LAP). It was noted that apoptotic cell engulfment leads to increased mitochondrial fission in macrophages, which was necessary for increased cytosolic levels of calcium, following LAP-associated calcium release from endoplasmic reticulum. In turn, cytosolic calcium was necessary for recycling of the phagolysomal membrane after the first round of phagocytosis and for complete sealing of the phagosome for engulfment of the cargo during the second LAP event. This enabled repeated uptake of apoptotic cells by the same macrophage[105]. Disabling mitochondrial fission inhibited repeated efferocytosis[105].

Identity of dead cells.

The identity of the dead cell may also determine the specific effector function. Although macrophages are generally thought to be agnostic to the identity of dying cells, macrophage engulfment specifically of dead type II alveolar epithelial cells in the lung has been identified as a trigger for lung fibrosis[106,107]. Similarly, dead cells can contain information regarding whether they died from an infection or otherwise. DC-mediated efferocytosis of uninfected apoptotic cells led to T_{reg} cell differentiation, whereas DC-mediated efferocytosis of infected apoptotic cells in the gut of mice infected with *Citrobacter rodentium* led to T_H17 cell differentiation[19]. These results indicate that DCs not only recognize uninfected

and infected apoptotic cells as distinct identities, but also respond in a manner that is specific and appropriate. These observations suggest that the information embedded in a dead cell likely extends beyond hallmarks such as PtdSer exposure and involves unique features, at least for some cells. These unique features, including pathogen-associated molecular patterns (PAMPs) or DAMPs, may engage different receptors in the efferocyte and underlie the distinct effector responses. Indeed, IL-1 family DAMPs (including IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ) can differentially direct T_H cell polarization to shape the adaptive immune response[108], and IL-18 or IL-33 can induce T_{reg} cells to produce amphiregulin and trigger lung tissue repair after influenza virus infection[109]. These cytokines function in sterile inflammation and also synergize with PAMPs to amplify infection-associated inflammation[108].

The role of the environment

The final factor to determine the response to cell death is the contextual frameworks in which cell death and its sensing occur. We posit that the tissue environment can directly regulate the effector response of the efferocyte either through specialized cytokines that are uniquely present in an environment, or through unique tissue-specific epigenetic and/or transcriptional changes or unique metabolic functions associated with a specific environment (FIG. 4). Integrating multiple coincidental signals in an environment-specific context could potentially limit the possible effector responses of macrophages from a broader generic repertoire to a specific subset of tasks. Finally, large-scale changes in effector functions and efferocytosis are observed in aging tissue.

Specialized cytokine environment.

The tissue environment can shape the response to dead cells by specifying the identity of the efferocyte, which in turn determines the effector response. For example, keratinocytes produce IL-34, which is required for the differentiation of Langerhans cells[110]. Langerhans cells are a locally self-renewing subset of DCs found in skin[111], together with other efferocytes such as dermal DCs, classical DCs and macrophages. Despite the presence of a large number of different efferocytes in skin, Langerhans cells specifically are necessary for the generation of antigen-specific-T_H17 cell responses[112]. By contrast, Langerin$^+$ dermal DCs are required for the generation of antigen-specific cytotoxic T lymphocytes[112].

Mammary stem cells were shown to require the support of macrophages recruited by colony-stimulating factor 1 (CSF1)[113]. Homozygous loss of function of CSF1 resulted in failure to recruit macrophages to developing mammary glands and a severe reduction in the ability of mammary stem cells to repopulate mammary glands[114]. Thus, CSF1-mediated recruitment of macrophages shapes mammary gland morphogenesis. Whether this effector function involves macrophage-mediated efferocytosis remains unknown; however, involution or apoptotic death of mammary epithelial cells and their removal by non-professional epithelial cells and professional macrophages are known to precede repopulation of the mammary gland by differentiation of mammary stem cells into adipocytes[115,116].

A more direct role of cytokines together with cell death sensing in shaping the expression of tissue-repair genes is shown by the example that macrophages trigger tissue repair upon

exposure to IL-4 together with apoptotic cells, but not IL-4 or apoptotic cells alone[18]. There are other examples of the effect of the tissue environment on macrophage functions, although in these cases the role of cell death was not explored. For example, surfactant protein A in the lungs following helminth-induced lung tissue damage, and C1q in the liver following *Listeria monocytogenes* infection, function as local amplifiers of IL-4-dependent macrophage-mediated tissue repair[117]. Comparing a biological material (urinary bladder matrix) with a synthetic material (polycaprolactone), it was shown that whereas urinary bladder matrix promotes tissue repair through a T_H2 cell- and IL-4-mediated programme, polycaprolactone drives $CD9^+IL\text{-}36\gamma^+$ macrophages towards a T_H17 cell- and IL-17-mediated programme, resulting in fibrosis[118].

Tissue-specific enhancers and transcription factors.

Tissue environments are well known to shape the distinct phenotypes of resident or recruited macrophage subsets by differentially regulating enhancer elements and the expression of transcription factors[119]. For example, monocytes recruited to the liver following Kupffer cell ablation differentiate into Kupffer cells under the combined influence of transforming growth factor-β and bone morphogenetic protein 2 produced by liver sinusoidal endothelial cells[120]. Similarly, imprinting of naive macrophages with the alveolar macrophage fate requires their spatial proximity with lung basophils[121]. Large cavity macrophages that populate peritoneal, pleural and pericardial spaces require a $WT1^+$ mesothelial and fibroblastic niche for their maintenance[122]. Thus, the dedicated effector functions of these specialized efferocytes are shaped by their tissue environment.

Intriguingly, at least in some cases, efferocytes remain capable of differential gene expression when removed from the specified environment. Lung alveolar macrophages were reported to be intrinsically less responsive to IL-4 compared with lung interstitial macrophages or peritoneal cavity macrophages[123], but lung alveolar macrophages can regain IL-4 responsiveness once removed from the lung environment[123]. The insensitivity to IL-4 was driven by the distinct metabolic state of lung alveolar macrophages in the lung environment[123].

Functional changes in the effector responses of aging macrophages may result from the epigenetic and/or metabolic alterations that occur in these cells. Commonly, pro-inflammatory cytokine production as well as MHC class II and CD86 expression are increased with aging[124,125]. Aged macrophages are also less effective as efferocytes[125]. Aging tissues are characterized by several generic features, including genomic instability, epigenetic changes, protein misfolding coupled with a reduced unfolded protein response, and autophagy and mitochondrial dysfunction[124,126]. Other mechanisms might also be involved. Reduced efferocytosis by aged macrophages correlated with a reduction in MERTK expression, through cleavage of this receptor[127]. This was associated with a skewing towards the production of leukotriene B_4 by macrophages instead of resolvin D1[127].

Commensals and other extrinsic signals.

The sensing of cell death by efferocytes is often integrated with external cues such as PAMPs and DAMPs. As described earlier, apoptosis in the absence of microbial signals induces T_{reg} cell differentiation, whereas apoptosis associated with *C. rodentium* infection resulted in T_H17 cell differentiation[19]. In addition, it was shown that phagocytosis of *Escherichia coli*-infected neutrophils by DCs lacking *Myd88* and *Trif* or *Tlr4,* which have defects in PAMP sensing, led to T_{reg} cell differentiation instead of T_H17 cell differentiation[19]. Another paradigm described earlier regarding plasticity between apoptosis and necroptosis during embryonic development can be further extended to include pyroptosis. Whereas *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ mice, which have defects in apoptosis and necroptosis, are viable[103], *Casp8*$^{C326A/C326A}$*Mlkl*$^{-/-}$ mice, which have a catalytically inactive caspase 8 mutation, die during perinatal development[128], at least in part owing to pyroptosis and inflammation-associated damage in the intestine[95,96]. Importantly, this phenomenon is restricted mostly to the gut, as endothelial cells or skin epithelial cells were not susceptible to pyroptosis. Thus, plasticity of cell death and the associated response might be determined through the integration of an environmental microbial signal. It will be interesting to learn if the pyroptotic death seen in *Casp8*$^{C326A/C326A}$*Mlkl*$^{-/-}$ intestine is also observed for mice bred in gnotobiotic facilities.

Conclusions and future directions

It remains a mystery how a unique effector response from amongst the multitude of possible effector responses to cell death is selected in response to a specific event of cell death to ensure that the physiological requirements of the tissue and/or organism are fulfilled. Here, we propose an integrated approach, primarily focusing on the efferocyte as a compiler of the source code that is written in the death of a cell. Thus, cell death undoubtedly contains invaluable information, but additionally requires an intermediate for the execution of the programme encoded by this information. We also include the environmental context as an additional factor that can influence the effector response by determining the form of cell death or by affecting the efferocyte. Together, these components define a cell death code that directs the effector functions associated with cell death (BOX 2).

What could be the importance of proposing this code of cell death? We believe that this theoretical construct can reveal unique specificities of the immune response to different inducers as well as explaining the dysregulation of the magnitude and resolution of the immune response when the code goes awry. For example, necroptosis has been proposed to evolve as a developmental checkpoint that aborts fetuses with severe developmental abnormalities[97]. This form of cell death also promotes adaptive immunity, for example during pneumococcal or viral infections[129,130]. Yet, necroptosis is associated with several diseases that might have in common an aetiology of dysregulated inflammation[97,131,132]. The improper execution and/or recognition of necroptosis may contribute to dysregulation of the magnitude of the immune response in relapsing–remitting diseases such as inflammatory bowel disease or even in neurodegenerative diseases.

Another potential outcome of a cell death code may be a principle that allows us to understand the difference in the type of immune response that is associated with, for

example, a viral infection compared with sterile tissue damage — in other words, the quality of the immune response. It is possible that a combination of the type of cell death and its recognition by efferocytes underlies at least some of the differences in the effector response in these settings. At least functionally, antigen presentation is a crucial discriminating factor between infection and sterile injury and therefore it is likely that the recognition of cell death by professional antigen-presenting cells such as DCs is more relevant in the context of an infectious injury, whereas macrophages may be more strongly associated with sterile injuries.

In cancer, malignant cells can die by apoptosis, necroptosis or ferroptosis (BOX 1). Furthermore, apoptotic cancer cells can be phagocytosed by DCs, monocyte-derived infiltrating macrophages, tissue-resident macrophages or even fibroblasts[133]. The consequences of efferocytosis of tumour corpses by DCs or macrophage subsets are not completely characterized. One study identified $CD103^+$ DCs, but not $CD11b^+$ DCs, as crucial effector cells for the uptake and presentation of tumour antigens for T cell priming in the lymph nodes and generation of tumour-infiltrating T cells[134]. Perhaps the redirection of efferocytosis towards $CD103^+$ DCs could be used to boost the anti-tumour response. Fibroblasts normally lack mechanisms for the presentation of antigens, although antigen presentation is upregulated under certain conditions such as in response to IFNγ signalling[135,136]. Thus, fibroblast-mediated phagocytosis of tumour corpses may lead to either immune evasion or the presentation of tumour-derived antigens, depending on the cytokine environment. Therefore, it is possible that the identity of the efferocyte and the environmental signals contribute to determining whether or not an anti-tumour immune response will be induced.

Changes to the cell death code — be the type of cell death, the efferocyte that encounters the dead cell or the cytokine environment, or an integration of several events — might also regulate the period of the immune response by encoding the timing of the transition from inflammation to resolution, which is a crucial step in the recovery of tissue function following injury. Insufficient, as well as excessive, tissue repair damages mucosal tissue and impairs gastrointestinal function in inflammatory bowel disease and other intestinal diseases such as radiation enteritis and chronic ischemic enteritis, ranging from ulcers, abscesses and fistulas to fibrosis and strictures[137]. Fibrosis in other organs such as the lung or the liver also presents significant clinical problems[138,139]. Cystic fibrosis is associated with bronchiectasis and is also known to cause colonic wall thickening, fibrotic colonopathy and strictures[137,140,141]. Chronic foot ulcers are a frequent complication of patients with long-term diabetes and up to 14–24% of these patients require amputations[142]. Therapeutic interventions to improve wound healing are extremely limited. Interestingly, the removal of dead cells (known as debridement) is one of the few known ways of improving the healing of non-infected wounds[143].

Discovering a fundamental, generalizable principle for the regulation of the magnitude, quality and period of the immune response will help us to better understand a vast array of diseases, including inflammation-associated degenerative diseases, diseases characterized by uncontrolled or error-prone immune responses and/or wound repair, as well as anti-cancer immunity. Recently, the plasticity between different modalities of cell death, such as upon

inhibition of caspase 8 enzymatic activity or its proteolytic processing, has been discovered[95,96]. Redirecting phagocytosis between effector cells may alter the rate of uptake of dead cells. Both microglia and astrocytes are capable of efferocytosis but microglia are three-times more efficient than astrocytes in the phagocytosis of apoptotic lymphocytes in vitro[144]. Furthermore, the efferocytosis rate of microglia can be modulated by cytokines — IFNγ stimulates efferocytosis whereas IL-4 reduces efferocytosis — whereas astrocyte efferocytosis is agnostic to cytokine exposure[144]. Perhaps, one day we will be able to toggle modes of cell death, the responding efferocytes and environmental signals for improved immune function in hitherto difficult to treat diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Glossary

LC3-associated phagocytosis (LAP)
A form of phagocytosis during which the canonical autophagy protein LC3 is conjugated to the phagosome to form the LAPosome. LC3 conjugation is crucial for phagosome maturation and acidification, through fusion with lysosomes, and the degradation of cargo

Necrosome complex
An amyloid signaling complex assembled upon interaction and activation (phosphorylation) of RIPK3 and RIPK1. The necrosome leads to the phosphorylation of MLKL and induction of necroptosis

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Box 1 | Overview of the major types of cell death and their molecular mechanisms

Here, we outline the major programmed cell death modalities discussed in this Review. For a more detailed description, we refer the reader to REF.[46].

Apoptosis

Stimuli inducing intrinsic apoptosis results in the formation of mitochondrial permeability transition pores and the release of cytochrome *c* into the cytoplasm. Cytochrome *c* binds to APAF1 inducing its oligomerization, thereby forming the apoptosome. The apoptosome activates the initiator caspase, caspase 9, which in turn activates the executioner caspases, caspase 3 and caspase 7. Cellular targets such as laminin, PARP and DNA are cleaved. Additionally, the release of SMAC/DIABLO from mitochondria neutralizes the suppressive effects of inhibitor of apoptosis proteins (IAPs), promoting the activation of executioner caspases. In extrinsic apoptosis, activation of death receptors of the tumour necrosis factor (TNF) family, including TNF receptor 1 (TNFR1), FAS (also known as CD95) and TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (also known as TNFRSF10A and TNFRSF10B), leads to formation of the death-inducing signalling complex (DISC), which activates the initiator caspase, caspase 8. Caspases cleave ATP11C, an enzyme that flips phosphatidylserine (PtdSer) from the outer leaflet to the inner leaflet of the plasma membrane and activate XKR8, an enzyme that moves PtdSer to the outer leaflet of the plasma membrane. This allows for exposure of PtdSer on the outer leaflet of the plasma membrane of apoptotic cells. PtdSer functions as an ‘eat me’ signal recognized by various receptors on neighbouring cells, thus promoting the engulfment of the apoptotic cell by the efferocyte.

Necroptosis

The activation of receptor-interacting serine/threonine-protein kinase 3 (RIPK3) by RIPK1, downstream of FAS, TNFR1, Toll-like receptor or interferon receptor signaling, drives necroptosis. The activation of RIPK3 by RIPK1 occurs when caspase 8 activation is suppressed and results in the formation of the necrosome complex **[G]**. RIPK3-dependent phosphorylation of mixed lineage kinase domain-like protein (MLKL) results in MLKL pore assembly at the plasma membrane. MLKL pores promote the flux of calcium, sodium and potassium ions. Necroptosis is generally associated with sterile inflammation. Several damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1) and ATP, are released during necroptosis. Additionally, inflammasome activation leads to release of proinflammatory cytokines and chemokines. Necroptotic cells are known to expose PtdSer and to be engulfed by macrophages as well as fibroblasts. It is unknown whether specific pathways exist for the recognition and clearance of necroptotic cells.

NETosis

In NETosis, the dying neutrophil releases a neutrophil extracellular trap (NET) composed of decondensed chromatin studded with anti-microbial proteins such as neutrophil elastase and myeloperoxidase (MPO) that traps and kills pathogens. Neutrophil elastase,

MPO and NADPH oxidase are required for the production of reactive oxygen species (ROS) and/or DNA decondensation during NET formation. NETosis can be stimulated by complement components and complement opsonization. In turn, NETs may activate complement as well as the clotting cascade and platelet aggregation. Removal of NETs requires macrophages.

Pyroptosis

Pyroptosis is observed primarily in macrophages and their precursors. Activation of caspase 1 and caspase 11 in mice results in the cleavage and activation of gasdermin D (GSDMD). The amino-terminal GSDMD fragment, GSDMD-N, oligomerizes to form pores in the plasma membrane. Various caspases cleave and enable the secretion of IL-1 family cytokines (such as IL-1β and IL-18) and also cause the release of DAMPs such as lactate dehydrogenase and HMGB1. Pyroptotic cells are known to expose PtdSer. Macrophages and fibroblasts have been described to engulf pyroptotic cells.

Ferroptosis

Ferroptosis is dependent on free iron or iron-containing lipoxygenase enzyme **[Au:OK?]** and is defined by the accumulation of ROS, the oxidation of polyunsaturated fatty acid-containing phospholipids and the loss of lipid peroxide repair capacity driven by phospholipid hydroperoxidase GPX4. PtdSer exposure has been described in ferroptosis and macrophages were shown to be capable of engulfing ferroptotic cells.

Necrosis

Necrosis is an accidental, passive cell death, such as when killed by heat or during osmotic lysis. The morphological hallmark of necrotic cells is rupture of the cell membrane, swelling of organelles and poring out of cytoplasmic components. Among the released substances are DAMPs that induce an inflammatory response. Macrophages have been shown to engulf necrotic cells.

Box 2 | A mathematical model for effector responses to cell death

Classical understanding of the effector response

Cell death can occur by multiple molecular mechanisms. Information regarding how the cell died is contained within the dead cell. In addition, there may be information regarding whether the cell is infected or uninfected, and even about its specific identity. Let $\mathcal{T}$ be the set of *'types of programmed cell death'*, let $\mathcal{I}d$ be the set of *'identities of the dying cell'* and let $\mathcal{O}$ be the set of *'other variables'*. Then, the set $\mathcal{D}$ of *'cell deaths'* can be described formally as the Cartesian product shown in equation (1.1).

$$\mathcal{D} := \mathcal{T} \times \mathcal{I}d \times \mathcal{O} \tag{1.1}$$

In other words, a type of *'programmed cell death'*, an *'identity of the dying cell'* and *'other variable'* completely characterize a type of *'cell death'*. The simplest response to cell death occurs where death itself executes the programmed function and, hence, the *'effector response'* is a direct function of *'cell death'*. Formally, if we denote $\mathcal{E}ff$ as the set of *'effector responses'*, then a type of *'cell death'* can give rise to exactly one type of *'effector response'* and thus be described as the function given in equation (1.2).

$$f : \mathcal{D} \rightarrow \mathcal{E}ff \tag{1.2}$$

That is, a type $d \in \mathcal{D}$ of *'cell death'* can be thought to be assigned to a unique type of *'effector response'* $f(d) \in \mathcal{E}ff$.

Proposed theory of the effector response

Our proposition is that the classical understanding of the *'effector response'* as a function of *'cell death'* given by equation (1.2) is incomplete because a type $d \in \mathcal{D}$ of *'cell death'* can be observed to give rise to multiple *'effector responses'*. Instead, we take into account the environment of cell death and the specific efferocyte involved in disposing of the dead cell, and we propose a new understanding of *'effector responses'* as a function of these additional inputs. More precisely, if we denote $\mathcal{E}nv$ as the set of *'environments of cell death'* and $\mathcal{E}ffero$ as the set of *'specific efferocytes'* then we propose the following new understanding of the *'effector response'* as a function of *'cell death'*, *'environment of cell death'* and *'specific efferocyte'* given by equation (2.1).

$$f : \mathcal{D} \times \mathcal{E}nv \times \mathcal{E}ffero \rightarrow \mathcal{E}ff \tag{2.1}$$

In other words, a type $d \in \mathcal{D}$ of *'cell death'*, a type $x \in \mathcal{E}nv$ of *'environments of cell death'* and a type $y \in \mathcal{E}ff$ of *'specific efferocyte'* give rise to a unique type $f(d, x, y) \in \mathcal{E}ff$ of *'effector response'*.

In some special cases, the environment of cell death and the type of cell death can be constrained, for example pyroptosis and inflammation are obligatorily paired. The environment may also pre-specify the type of efferocyte that is available to recognize and dispose of the dead cell. These constraints can be considered by a simple extension of

equation (2.1). Namely, there is a subset $\mathscr{A} \subset \mathscr{D} \times \mathscr{E}nv \times \mathscr{E}ff ero$, which we refer to as the set of *'admissible combinations',* for which the *"effector response"* can be understood as a function of the set of *'admissible combinations'*, as given by equation (2.2).

$$f : \mathscr{A} \rightarrow \mathscr{E}ff \tag{2.2}$$

In other words, in equation (2.2), a type $d \in \mathscr{D}$ of *"cell death"*, a type $x \in \mathscr{E}nv$ of *'environments of cell death'* and a type $y \in \mathscr{E}ff$ of *'specific efferocyte'* give rise to a unique type $f(d, x, y) \in \mathscr{E}ff$ of *'effector response'* if the triple $(d, x, y) \in \mathscr{A}$ is an *'admissible combination'*.

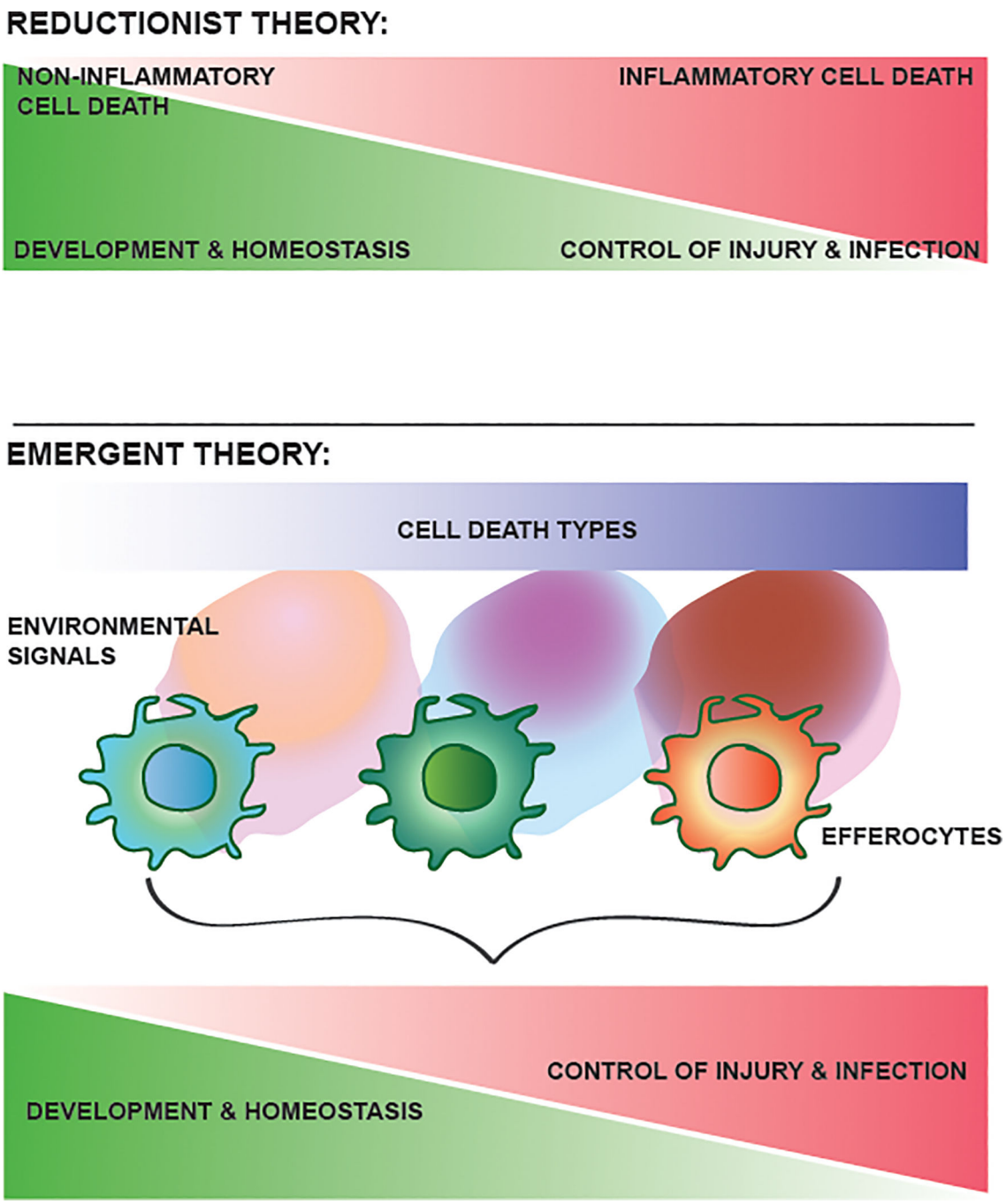

Figure 1 |. Schematic representation of the proposed code of cell death.

According to a reductionist theory (top), there is a one-to-one relationship between the type of cell death and the effector function. As an example, non-inflammatory cell death is associated with physiological resolution and repair responses, whereas inflammatory cell death is associated with the control of injury and infection but can result in pathologies associated with defective resolution or repair. By contrast, the emergent theory (bottom) that we outline in this Review takes into account the variety of cell death types, the multitude of accompanying environmental signals and their integration by different efferocytes for the

execution of specific effector responses. As such, the same cell death modality can result in distinct effector functions, depending on the specificity of environmental signals and the efferocyte. For simplicity, only three types of cell death, environment, efferocyte and effector response are shown here, but in the broader contexts of development, homeostasis and repair, there are a large set of spatially and temporally distinct combinations resulting in different effector responses.

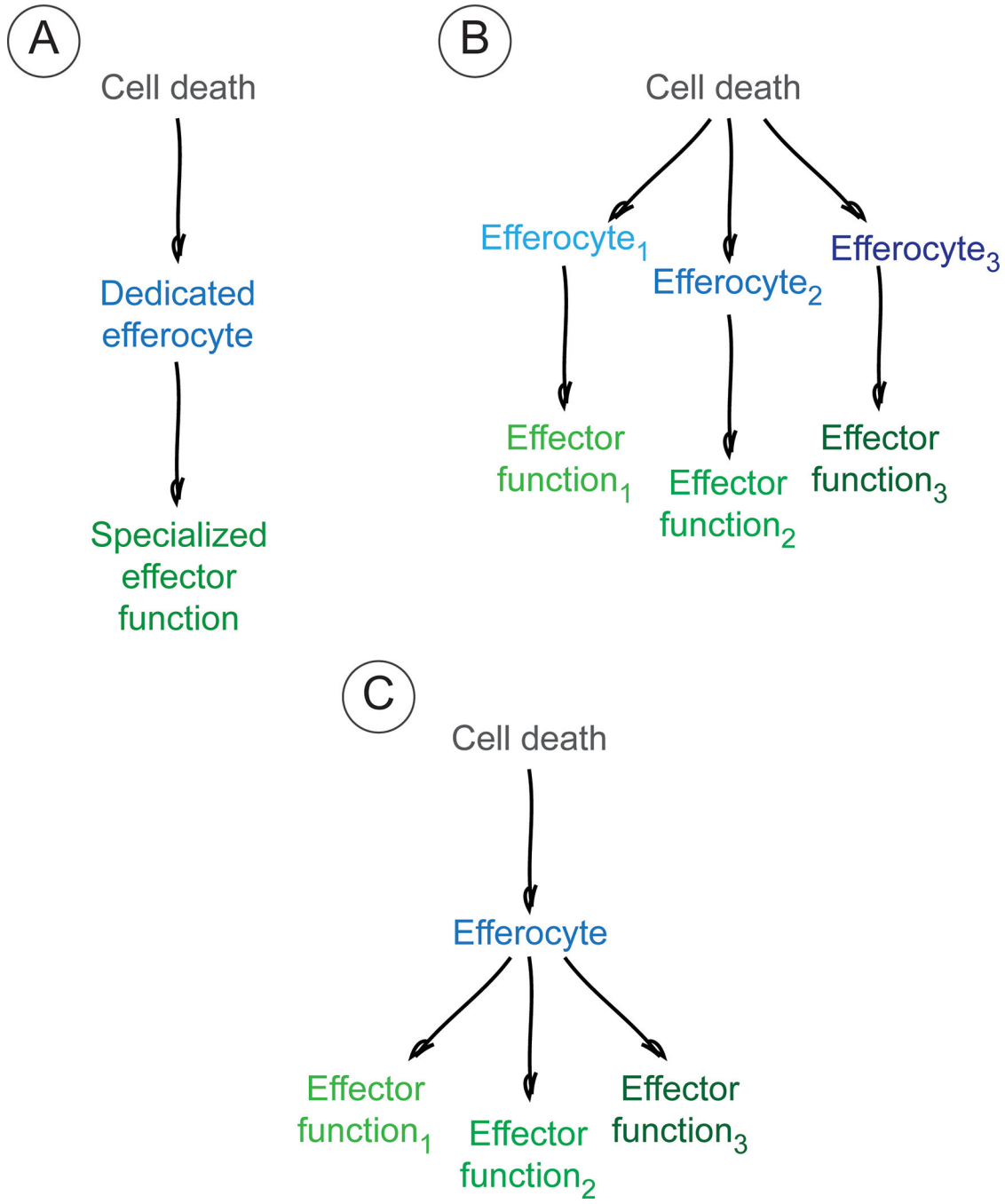

Figure 2 |. The role of the efferocyte.

Three distinct scenarios represent possible relationships between cell death, efferocytes and effector responses. **a** | Recognition of a particular type of dead cell by a dedicated efferocyte results in a specialized effector function. **b,c** | Alternatively, the recognition of dead cells could give rise to multiple and distinct effector functions. This could result from the recognition of dead cells by different efferocytes (**b**) or from divergent effector responses being executed by the same efferocyte (**c**).

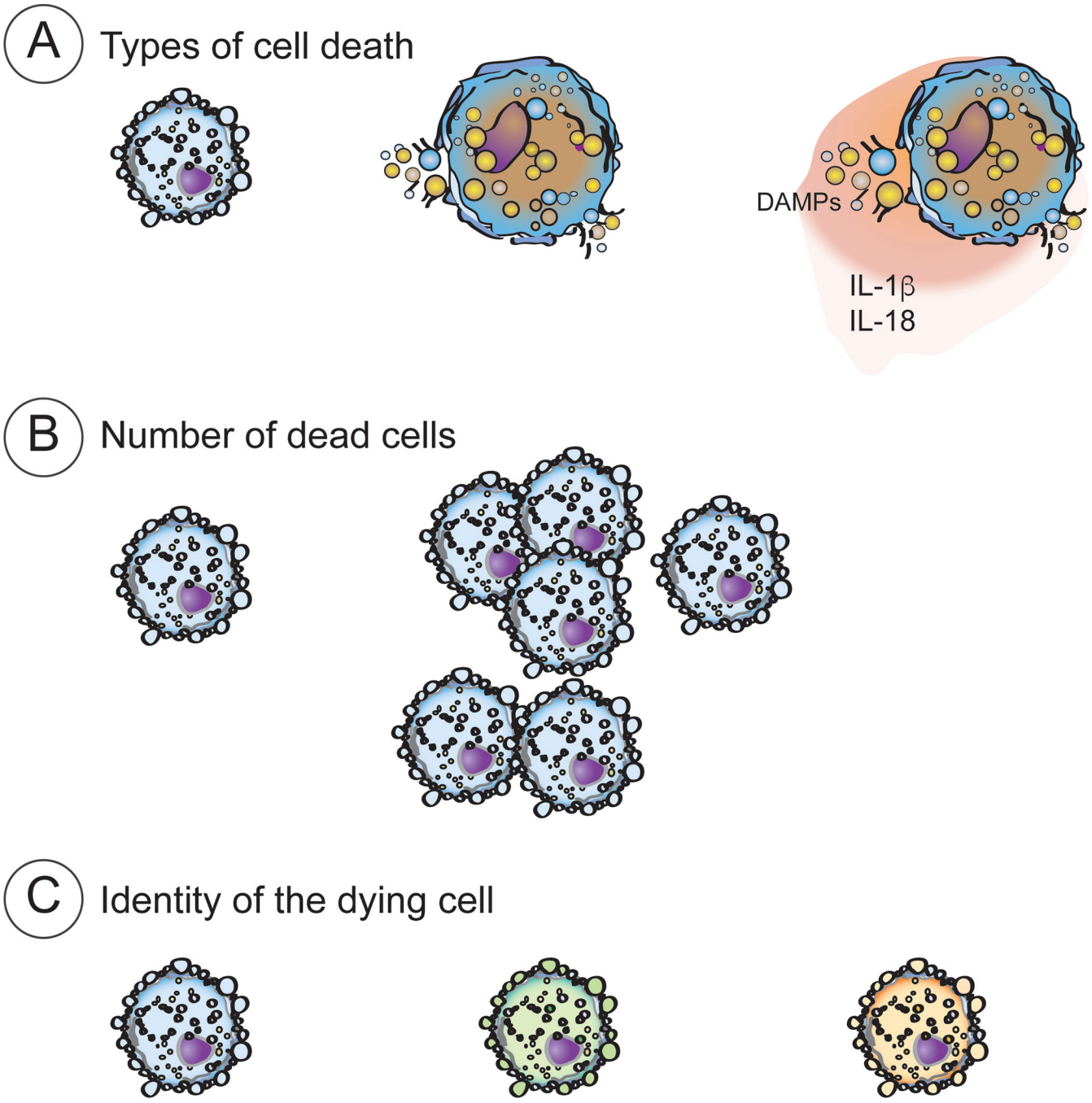

Figure 3 |. The role of the dying cell.

The information provided by the dying cell can be contained in the type of cell death modality, the number of dead cells and the identity of the dead cells.

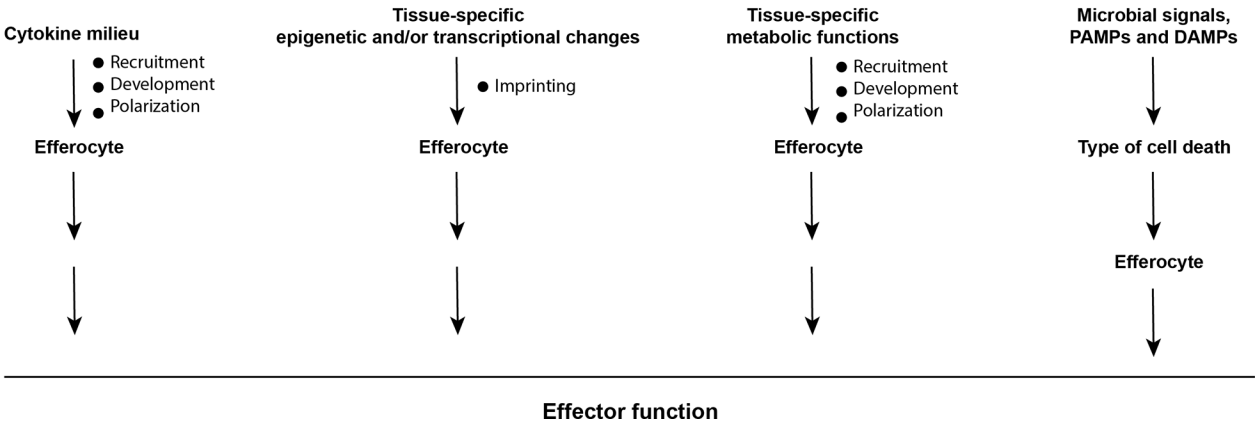

Figure 4 |. The role of the environment.

The tissue environment can contribute to determining the effector response of the efferocyte either directly through specialized cytokines that are uniquely present in an environment, or through unique tissue-specific epigenetic and/or transcriptional changes or unique metabolic functions associated with a specific environment, or indirectly by influencing the type of cell death, which in turn can affect the effector response.