CELL DEATH AND IMMUNITY

TIMELINE

Programmed cell death and the immune system

Shigekazu Nagata and Masato Tanaka

Abstract | More than 50 years ago, cells were observed to die during insect development via a process that was named 'programmed cell death'. Later, a similar cell death process was found to occur in humans, and the process was renamed 'apoptosis'. In the 1990s, a number of apoptosis-regulating molecules were identified, and apoptosis was found to have essential roles in the immune system. In this Timeline article, we highlight the key events that have demonstrated the importance of programmed cell death processes, including apoptosis and programmed necrosis, in the immune system.

The adaptive immune system has evolved a unique strategy for protecting us against every possible pathogen that we encounter during our lifetime. In developing lymphocytes, the genes that encode antigen receptors are randomly rearranged to generate an extremely large repertoire of immune cells that respond to various pathogens and foreign materials. However, this system also has the potential to produce immune cells that react to self-antigens. To avoid this situation, the acquired immune system has developed multiple mechanisms for efficiently deleting self-reactive immune cells. In addition, activated immune cells need to be eliminated soon after they have accomplished their task in order to prevent excessive immune reactions that can cause host pathology. Furthermore, during an infection, pathogen-infected cells actively kill themselves before the pathogens multiply and kill cells in order to spread. In each of these immunological settings, programmed cell death has an essential role. In this Timeline article, we focus on the key studies that have helped us to understand the various functions of programmed cell death in the immune system (FIG. 1).

Apoptosis in the immune system

In 1965, Lockshin and Williams[1] discovered that specific cells die during the metamorphosis of the silkworm and designated this type of cell death 'programmed' because these cells were destined to die according to a 'construction manual' for the insect. In 1972, Kerr *et al.*[2] observed a specific type of cell death in human tissues in which the cells and nuclei became condensed and fragmented, and they called this cell death process 'apoptosis'. They proposed that apoptosis is crucial for regulating cell populations during tissue development and turnover. At around the same time, cytotoxic T lymphocytes (CTLs) were shown to induce cell death in their target cells or in virus-infected cells[3] (FIG. 2). Around 1990, apoptosis or cell death was reported to have important roles in the adaptive immune system in the deletion of thymocytes that express autoreactive or non-reactive T cell receptors (TCRs)[4,5], and in the deletion of autoreactive immature B cells[6,7]. The resolution of inflammation was also shown to be accompanied by the presence of dying neutrophils with an apoptotic morphology[8].

Two major apoptosis pathways

In the early 1990s, FAS ligand (FASL; also known as TNFSF6)[9] and FAS (also known as TNFRSF6, CD95 and APO1)[10] were identified as a death factor and its receptor, respectively. These findings, along with the discoveries of many other apoptosis-regulating genes in *Caenorhabditis elegans*[11,12], fruitflies[13] and mammals[14–17], revealed the existence of two major signalling pathways in apoptosis (FIG. 3). In one pathway, known as the 'death receptor' or 'extrinsic' pathway of apoptosis, the binding of FASL to FAS causes a conformational change of the FAS trimer. FAS then forms a multiprotein complex (known as the death-inducing signalling complex (DISC)) with the adaptor protein FAS-associated death domain protein (FADD) and pro-caspase 8 (REFS 18–20). Pro-caspase 8 is processed into mature caspase 8 in the DISC, and the activated mature caspase 8 then processes pro-caspase 3 to form mature caspase 3.

In the second pathway, which is called the 'intrinsic' pathway of apoptosis, a developmental signal or genotoxic agent activates a pro-apoptotic member of the B cell lymphoma 2 (BCL-2) family. The pro-apoptotic members of BCL-2 family stimulate mitochondria to release many molecules that can regulate apoptosis[21]. Among these molecules, cytochrome *c* forms a multiprotein complex (known as the apoptosome) together with pro-caspase 9 and apoptotic protease-activating factor 1 (APAF1), and the complex processes pro-caspase 9 to form mature caspase 9 (REFS 15,16,22). The activated caspase 9 then processes pro-caspase 3 to form mature caspase 3.

Caspase 3, which is activated in both the extrinsic and intrinsic pathways, subsequently cleaves more than 500 cellular substrates to execute the apoptosis programme. The involvement of cytochrome *c* and APAF1 in apoptotic cell death *in vivo* was confirmed in mice[23–25] and in *Drosophila melanogaster*[26]. However, these studies also revealed cytochrome *c*-independent or APAF1-independent activation of caspases 3 and 9, which suggests that other unidentified pathways also lead to caspase activation.

Defects in apoptosis result in autoimmunity. The spontaneous mouse mutations *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) result in lymphadenopathy and splenomegaly, and animals with these mutations show accelerated development of systemic lupus

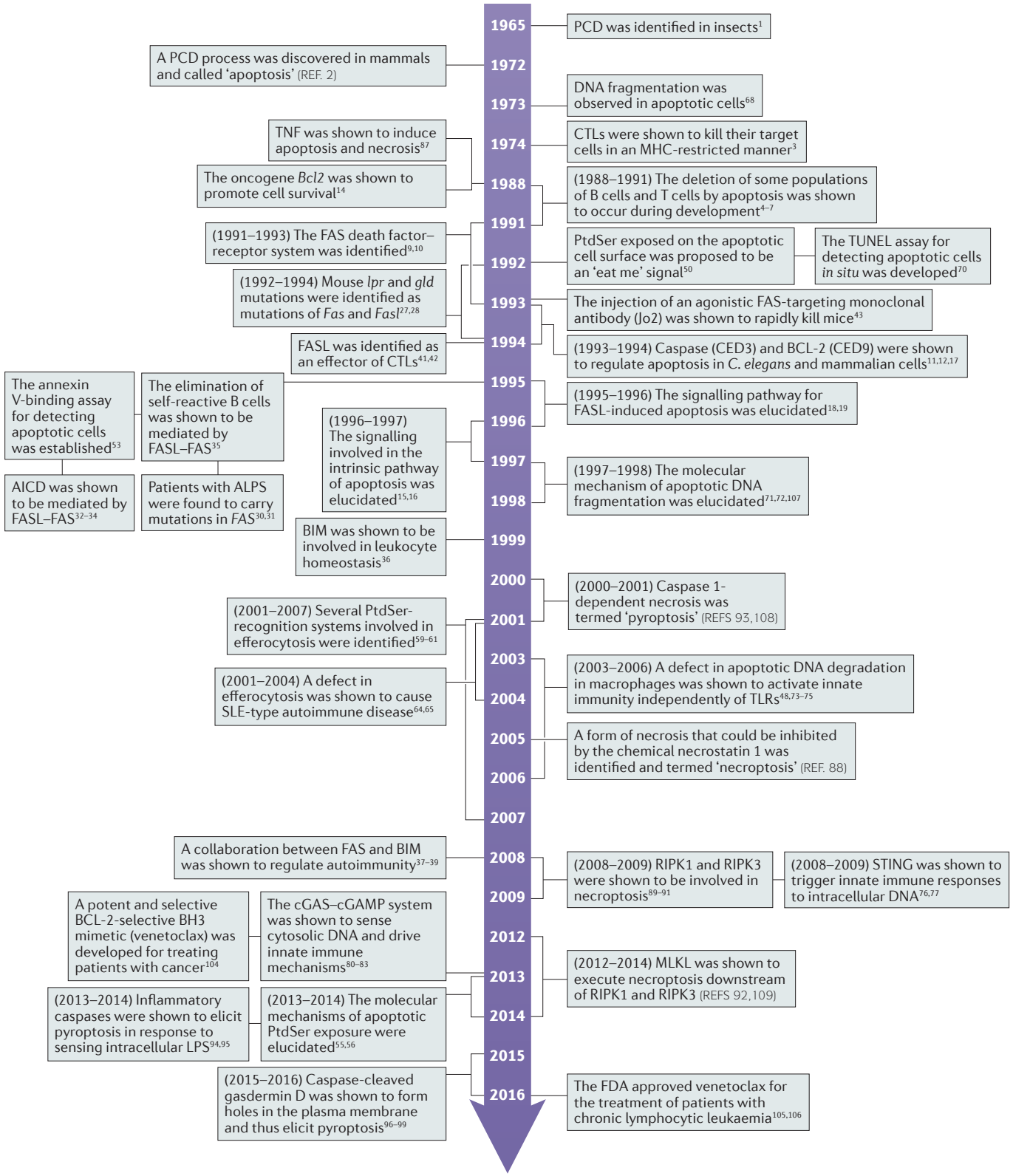

Figure 1 | **Timeline of the history of cell death research in immunology.** AICD, activation-induced cell death; ALPS, autoimmune lymphoproliferative syndrome; BCL-2, B cell lymphoma 2; BH3, BCL-2 homology 3; *C. elegans*, *Caenorhabditis elegans*; CED3, *C. elegans* homologue of mammalian caspases; CED9, *C. elegans* homologue of mammalian BCL-2; cGAMP, cyclic GMP–AMP; cGAS, cGAMP synthase; CTL, cytotoxic T lymphocyte; FASL, FAS ligand; FDA, US Food and Drug Administration; *gld*, generalized lymphoproliferative disease; *lpr*, lymphoproliferation; LPS, lipopolysaccharide; MLKL, mixed lineage kinase domain-like protein; PCD, programmed cell death; PtdSer, phosphatidylserine; RIPK, receptor-interacting protein kinase; SLE, systemic lupus erythematosus; STING, stimulator of interferon genes protein; TLR, Toll-like receptor; TNF, tumour necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

erythematosus (SLE)-type autoimmune diseases. Notably, the *lpr* and *gld* phenotypes were found to be caused by loss-of-function mutations in *Fas* (*Fas*^{lpr}) and *Fasl* (*Fasl*^{gld}), respectively[27,28]. Later, it was shown in humans that patients with autoimmune lymphoproliferative syndrome also carry somatic or germline mutations in the genes that encode FAS or FASL[29–31]. These results, together with the finding that the FASL–FAS system is indispensable for activation-induced cell death in T cells[32–34], suggested that the FAS-mediated extrinsic apoptosis pathway is responsible for the deletion of peripheral T cells. The *lpr* and *gld* mice also produce autoreactive antibodies, and it was found that the FAS system has a role in eliminating autoreactive B cells[35].

Mice deficient in *Bim* (also known as *Bcl2l11*), a pro-apoptotic member of the BCL-2 family that is involved in the intrinsic apoptotic pathway, also develop lymphoproliferation and suffer from an SLE-type autoimmune disease that is similar to the disease seen in *lpr* mice[36]. This suggested that the intrinsic and extrinsic apoptotic pathways have collaborative roles in maintaining lymphocyte homeostasis. Indeed, this collaboration was confirmed by studies showing that *Bim*-deficient *lpr* mice developed a more severe autoimmune phenotype than did either *Bim*-deficient mice or *lpr* mice[37–39]. In addition, a recent study found that BIM-mediated apoptosis is involved in regulating the lifespan of short-lived myeloid cells such as eosinophils, neutrophils and monocytes[40].

Expression of FAS and FASL. FASL expression is restricted to specific lymphocyte populations, such as CTLs, T helper 1 (T_H1) cells and natural killer cells[41,42]. By contrast, FAS is widely expressed by most cell types in various tissues, and the systemic injection of an agonistic FAS-targeting antibody or recombinant FASL quickly kills mice by inducing fulminant hepatitis[43,44]. Under physiological conditions, FASL-mediated apoptosis maintains tissue homeostasis, for example, by inducing cell death to resolve post-injury fibrosis or to prevent excessive myofibroblast proliferation in the lung[45]. Furthermore, based on findings from experiments that used a mouse model of allogeneic bone marrow transplantation, Tsukada *et al.*[46] proposed that FASL is involved in the development of graft-versus-host disease, whereas perforin produced by CTLs can mediate the graft-versus-leukaemia effect. If this relationship holds true in humans, blocking the FAS death system could be beneficial for patients who are undergoing cancer therapies and require bone marrow transplantation.

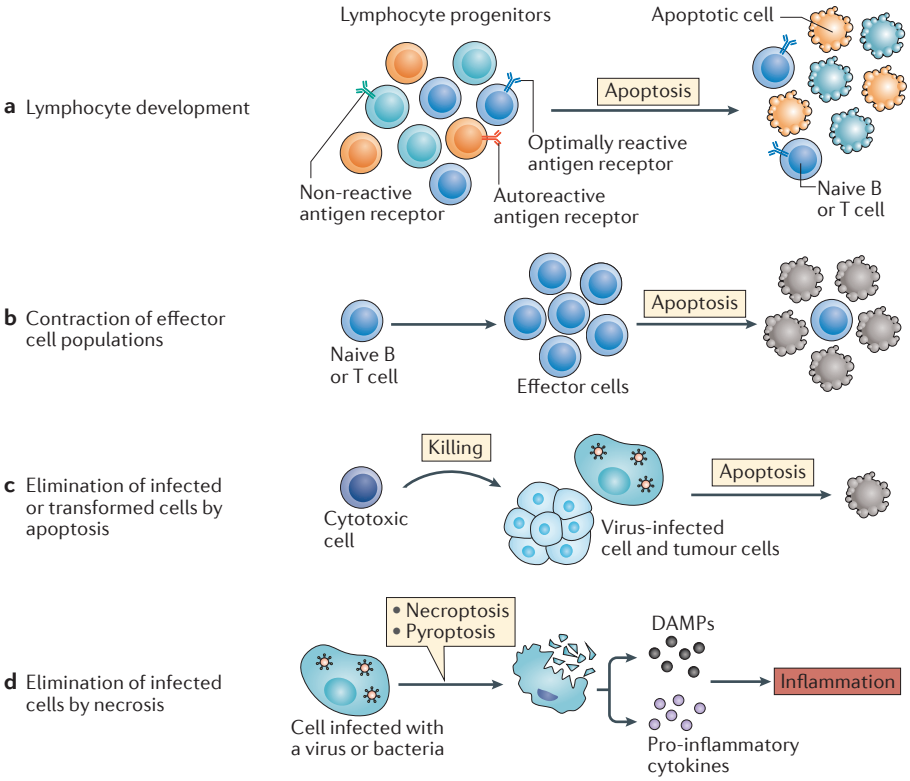

Figure 2 | **Cell death in immunological processes.** Cell death is involved in various immunological processes. **a** | During lymphocyte development, a large number of lymphocyte progenitors that express antigen receptors with a high affinity for self-antigens or that cannot respond to antigens are eliminated by apoptosis. The lymphocytes that survive this stage of development remain in the periphery and form the naive T cell and naive B cell compartments. **b** | When naive lymphocytes encounter pathogens, they proliferate and are activated to combat the pathogens. These activated lymphocytes will subsequently die after the pathogens have been removed. A similar situation can be found with neutrophils during inflammation (not shown). That is, when our body is infected by bacteria, neutrophil populations expand, and neutrophils are activated to phagocytose bacteria, but they quickly undergo apoptosis after the infection has been cleared. **c** | Cytotoxic T lymphocytes and natural killer cells recognize virus-infected, bacteria-infected and transformed cancer cells, and induce these cells to die by apoptosis. **d** | Bacteria-infected cells, particularly phagocytes, often undergo necrosis to prevent bacteria from proliferating further inside the cell. Unlike apoptosis, necrosis is an inflammatory form of cell death and can lead to further tissue inflammation. DAMPs, damage-associated molecular patterns.

The 'eat me' signal

In their landmark article on apoptosis, which was published in 1972, Kerr *et al.*[2] mentioned that although large numbers of cells die by this process, no inflammation or tissue destruction is observed. They found that almost all of the dead cells were inside 'histiocytes', indicating that apoptotic cells are quickly engulfed by phagocytes. This rapid phagocytosis of dying cells prevents inflammation that could be caused by noxious materials released from the dead cells. This process is now referred to as 'efferocytosis' to emphasize its uniqueness as a form of phagocytosis[47]. The observation of undigested apoptotic nuclei in the tissue macrophages of mice deficient in DNase II, a lysosomal nuclease, indicated that tissue-resident macrophages are the major players in efferocytosis[25,48]. Fibroblasts and endothelial cells may also carry out efferocytosis in macrophage-deficient tissues, but the efficiency of engulfment by these 'non-professional' phagocytes is low[49].

The realization that macrophages engulf apoptotic cells, but not healthy live cells, led to the identification of 'eat me' signals that are exposed by apoptotic cells. In 1992, Fadok *et al.*[50] reported that phosphatidylserine (PtdSer) exposed on the surface of apoptotic cells triggers efferocytosis. This finding, together with the observations that PtdSer is localized to the inner leaflet of the plasma membrane in healthy cells

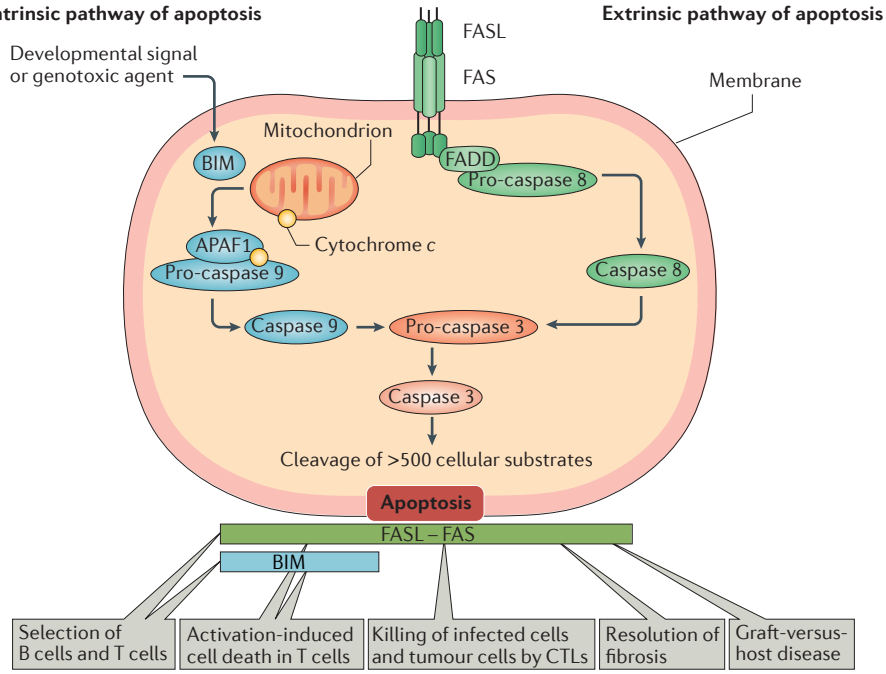

Figure 3 | **The two major apoptotic pathways.** In the extrinsic pathway of apoptosis, a death-inducing factor such as FAS ligand (FASL) binds its receptor (FAS) and recruits the adaptor FAS-associated death domain protein (FADD) and pro-caspase 8 to form the death-inducing signalling complex (DISC). The cleavage and activation of pro-caspase 8 in the DISC then activates a downstream caspase cascade that typically involves caspase 3. In the intrinsic pathway of apoptosis, a developmental programme or genotoxic agent activates a B cell lymphoma 2 homology 3 (BH3)-only protein, such as BIM, which stimulates the release of cytochrome *c* from mitochondria. Cytochrome *c* promotes the assembly of the apoptosome, which is a heptameric complex that comprises apoptotic protease-activating factor 1 (APAF1), pro-caspase 9 and cytochrome *c*. Mature caspase 9 generated by the apoptosome then cleaves pro-caspase 3 to form mature active caspase 3. Thus, both the extrinsic and intrinsic apoptotic pathways lead to the activation of caspase 3, which cleaves more than 500 cytoplasmic proteins to induce apoptotic cell death. The intrinsic and extrinsic pathways of apoptosis are involved in various immunological processes, including the selection of lymphocytes, activation-induced cell death in T cells, the killing of infected cells and tumour cells, the resolution of fibrosis and graft-versus-host disease. CTL, cytotoxic T lymphocyte.

and that PtdSer-overloaded red blood cells are recognized by macrophages[51], strongly suggested that PtdSer functions as an 'eat me' signal. Meanwhile, several groups identified molecules that are required for efferocytosis, some of which recognized PtdSer on apoptotic cells (see below). Masking PtdSer inhibits efferocytosis[52], which confirms that PtdSer is the major, if not unique, 'eat me' signal (FIG. 4).

PtdSer exposure is now known to be a hallmark of apoptosis, and annexin V, which specifically binds to PtdSer, is used as a marker of apoptotic cells[53]. The exposure of PtdSer depends on caspase activation[54], and recent studies from our laboratory have revealed precisely how PtdSer is exposed on the cell surface during apoptosis[55,56]. In healthy cells, ATP11A and ATP11C, which are phospholipid-transporting ATPases at the plasma membrane, actively translocate or flip PtdSer from the outer leaflet to the inner leaflet to confine PtdSer to the inner leaflet of the plasma membrane. In cells undergoing apoptosis, active caspase 3 cleaves and inactivates these ATPases. At the same time, XK-related protein 8 (XKR8), a transmembrane protein, is cleaved by caspase 3 at its carboxy-terminal tail region and functions as a phospholipid scramblase to scramble phospholipids between the inner and outer plasma-membrane leaflets, thus quickly exposing PtdSer.

Efferocytosis

In 1996 and 1997, a Japanese group[57,58] reported that a protein encoded by *Gas6* (growth arrest-specific protein 6) specifically recognizes PtdSer and functions as a ligand for MER, which is a member of the TYRO3, AXL and MER (TAM) tyrosine kinase receptor family. Subsequently, Scott *et al.*[59] found that MER-deficient macrophages have a defect in efferocytosis. Meanwhile, by establishing monoclonal antibodies that inhibit efferocytosis, our group identified two families of molecules that specifically recognize PtdSer on apoptotic cells (namely, the MFGE8 (lactadherin)–DEL1 (EDIL3) family, and the T cell immunoglobulin and mucin domain-containing protein 4 (TIM4)–TIM1 family)[60,61]. MFGE8 is a soluble protein that is secreted by certain macrophages and serves as a bridging molecule to bring apoptotic cells to integrin-expressing macrophages, and TIM4 and TIM1 are type I membrane proteins that function as PtdSer receptors. Efferocytosis was previously divided into two steps: namely, the 'tethering' and 'tickling' steps[62]. The tethering step involves the recruitment of apoptotic cells to phagocytes, whereas the tickling step involves the engulfment of apoptotic cells. A recent study by Nishi *et al.*[63] indicated that TIM4 and MER are involved in the tethering and tickling processes, respectively. A deficiency of MFGE8 or TAM receptors causes SLE-type autoimmune disease in autoimmunity-prone mice, which confirms that efferocytosis is important for tissue homeostasis[64,65]. As efferocytosis is accompanied by the production of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor-β (TGFβ)[66], it is possible that when efferocytosis is disrupted, the loss of these anti-inflammatory mediators and the exposure of immune cells to the intracellular components from unengulfed lysed cells contribute to the development of autoimmune diseases.

Degradation of dead cells

After being engulfed by macrophages, apoptotic cells are transported to lysosomes, where their components are degraded into building units (amino acids, nucleotides and monosaccharaides) for re-use. If this process does not proceed efficiently, it will cause a type of lysosomal storage disease[67]. Among the dead-cell components, the degradation of DNA in apoptosis has been well studied.

Early in apoptosis research, apoptosis was found to be accompanied by the fragmentation of chromosomal DNA into nucleosomal units[68,69], and this discovery led to the development of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay for detecting apoptotic cells[70]. Almost 20 years later, a caspase 3-activated endonuclease named caspase-activated DNase (CAD; also known as DNA fragmentation factor) was identified[71,72], and this enzyme was found to be solely responsible for cell-autonomous apoptotic DNA fragmentation[48]. The

fragmented DNA of apoptotic cells is further degraded by the endonuclease DNase II in the lysosomes of macrophages after the dead cells are engulfed. Thus, CAD-deficient mice have no physiological abnormalities, whereas DNase II-deficient mice have numerous abnormal macrophages that are filled with the undigested DNA of engulfed apoptotic cells[48]. These macrophages carrying undigested DNA produce interferon-β (IFNβ) and tumour necrosis factor (TNF) — which cause lethal anaemia and polyarthritis, respectively[73,74] — by a mechanism that is independent of Toll-like receptors (TLRs)[75].

Meanwhile, Ishikawa and Barber[76,77] found that stimulator of IFN genes protein (STING) in the endoplasmic reticulum mediates IFN production in response to intracellular viral DNA. By contrast, absence in melanoma 2 (AIM2) was shown to mediate the production of IL-1β in response to intracellular DNA via the activation of inflammasomes[78,79]. Subsequently, a series of elegant biochemical studies by Chen's group[80–83] showed that cyclic GMP–AMP (cGAMP) is synthesized by a specific enzyme (cGAMP synthase (cGAS)) that recognizes intracellular DNA and binds STING to induce the expression of IFN genes. Notably, deficiency of *Sting* or *Cgas* rescues the lethality of *Dnase2* deficiency in mice and blocks the development of polyarthritis[84,85]. A null mutation in *Aim2* also blocks the development of arthritis in *Dnase2*-deficient mice, albeit weakly[86]. These findings indicated that in *Dnase2*-deficient mice, the DNA of dead cells can leak from lysosomes into the cytoplasm, leading to the activation of the cGAS–STING and AIM2–inflammasome pathways, and induce the innate immunity. In other words, if the DNA of our own dead cells is not properly degraded, it behaves like a pathogen.

Immunogenic cell death

When apoptotic cells are not swiftly engulfed by macrophages, they undergo secondary necrosis during which they swell and the plasma membrane ruptures. In addition to this passive necrotic death, cells can die by programmed necrosis. TNF is one of the inflammatory cytokines that is produced by macrophages infected by a virus or by bacteria, and it usually stimulates the expression of genes that promote inflammation. However, TNF also has the ability to induce apoptosis or necrosis, particularly when it functions in the presence of inhibitors of protein synthesis or RNA synthesis[87]. The molecular mechanism of TNF-induced apoptosis is similar to that identified for FASL-induced apoptosis. However, TNF kills cells by inducing necrosis when the apoptosis pathway is inhibited. In 2005, Degterev *et al.*[88] designated the necrosis induced by TNF in the presence of a caspase inhibitor as 'necroptosis' and identified a compound (necrostatin 1) that inhibits this cell death process. The same group subsequently identified receptor-interacting protein kinase 1 (RIPK1) as the target of necrostatin 1 (REF. 89). In necroptosis, the binding of TNF to its receptor stimulates the kinase activity of RIPK1, which activates another kinase, RIPK3 (REFS 90,91). RIPK3 then phosphorylates mixed lineage kinase domain-like protein (MLKL), and the phosphorylated MLKL translocates to the plasma membrane, which it damages

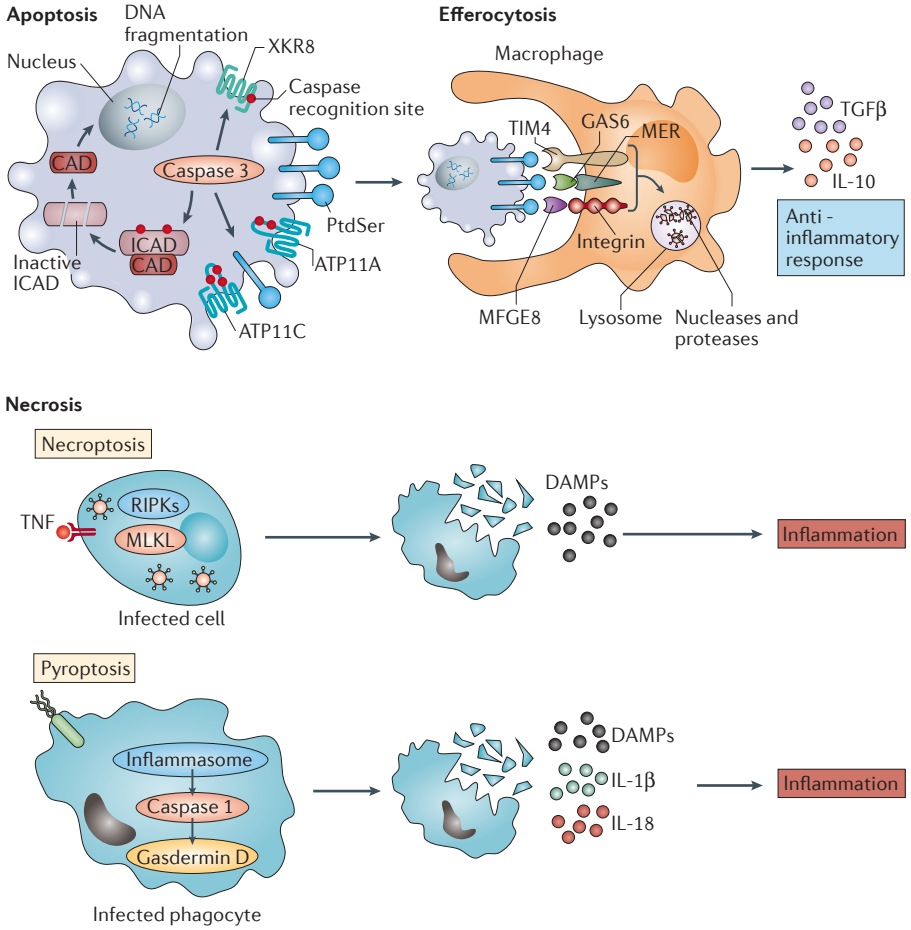

Figure 4 | **Apoptosis and programmed necrosis.** During apoptosis, activated caspase 3 cleaves inhibitor of caspase-activated DNase (ICAD; also known as DNA fragmentation factor subunit-α), thereby releasing caspase-activated DNase (CAD) to initiate DNA fragmentation. Caspase 3 cleaves the phospholipid flippases ATP11A and ATP11C and the phospholipid scramblase XK-related protein 8 (XKR8) to expose phosphatidylserine (PtdSer) as an 'eat me' signal on the cell surface.The exposed PtdSer is recognized by molecules such as MFGE8, growth arrest-specific protein 6 (GAS6) and T cell immunoglobulin and mucin domain-containing protein 4 (TIM4), and its recognition promotes efferocytosis by macrophages. The efferocytosed dead cells are transported into lysosomes where dead cell components are degraded by proteases and nucleases. Macrophages that have engulfed apoptotic cells produce transforming growth factor-β (TGFβ) and interleukin-10 (IL-10) to block inflammation. Necroptosis and pyroptosis are programmed forms of necrosis. In necroptosis, tumour necrosis factor (TNF) binds to TNF receptor 1 (TNFR1) in bacteria-infected or virus-infected cells, and activates receptor-interacting protein kinase (RIPKs). The activated RIPKs phosphorylate mixed lineage kinase domain-like protein (MLKL), which damages the plasma membrane, leading to the release of damage-associated molecular patterns (DAMPs). In pyroptosis, the intracellular DAMPs or bacterial endotoxins activate inflammasomes in phagocytes. Inflammasomes activate inflammatory caspases (caspase 1, caspase 4 and caspase 5 in humans; caspase 1 and caspase 11 in mice) to cleave gasdermin D, and the cleaved gasdermin D forms holes in the plasma membrane, which leads to the release mature IL-1β, IL-18 and DAMPs. In necrosis, DAMPs released from dead cells, IL-1β and IL-18 recruit macrophages, neutrophils and lymphocytes, and activate them to produce pro-inflammatory cytokines.

to execute necrosis[92]. TNF does not normally kill cells; however, when cells are infected by a virus or by bacteria, their transcriptional or translational machinery is inhibited, which sensitizes the cells to TNF-induced cytotoxicity. Furthermore, viruses and bacteria often encode molecules that inhibit apoptosis. Thus, cells infected by a virus or by bacteria undergo necroptosis upon their engagement with TNF.

Another type of programmed necrosis is pyroptosis, a term coined by Cookson and Brennan[93] in 2001 to distinguish the caspase 1-dependent necrosis of macrophages infected with *Salmonella* spp. from the passive or accidental necrotic process. Two pathways, which are referred to as the canonical and non-canonical pathways, are known to execute pyroptosis. In the canonical pathway, molecules associated with pathogens or released from dead cells stimulate the formation of inflammasomes, which are multiprotein complexes that mediate the processing and activation of pro-caspase 1. In the non-canonical pathway, endotoxins from Gram-negative bacteria directly bind to human pro-caspase 4 or pro-caspase 5 (or their mouse homologue, pro-caspase 11), and activate these caspases[94,95]. In both the canonical and non-canonical pathways of pyroptosis, caspase 1, caspase 4 or caspase 5 cleaves a protein called gasdermin D, and processes pro-IL-1β and pro-IL-18 into their mature forms[96,97]. The approximately 30 kDa amino-terminal domain of gasdermin D then translocates to the plasma membrane, where it forms holes that cause necrosis and enable the release of the processed mature forms of IL-1β and IL-18 (REFS 98,99).

Thus, pyroptosis has an important role in triggering inflammation by releasing IL-1β and IL-18. In addition, pyroptosis and necrosis are involved in killing pathogen-infected cells before the invading virus or bacteria proliferate[90,100] (FIG. 2). These necrotic processes are accompanied by the release of cellular contents from the dying cells, which act as damage-associated molecular patterns (DAMPs) to stimulate pro-inflammatory processes, including the recruitment and activation of neutrophils, macrophages and other immune cells. Thus, although these necrosis systems combat pathogens, they also lead to strong, tissue-damaging inflammation and, as described above for defective efferocytosis, the chronic exposure of DAMPs to the immune system may drive autoimmunity (FIG. 4).

Conclusion

Here, we have briefly reviewed the history of the field of programmed cell death. Since the identification of apoptosis-mediating molecules in the early 1990s, this field has seen tremendous progress. In 1990, we did not know what triggered apoptosis, how the cells died or how macrophages recognized apoptotic cells to swiftly clear them away. We now know — or at least have a good idea — about the cellular and molecular mechanisms that underlie apoptosis. In addition, we have learned that necrosis, which used to be considered as an accidental passive death process, can be programmed, and molecules that can execute this process have been proposed. As outlined in BOX 1, a number of key questions remain. There are many human diseases that are associated with an abnormal cell death process. More detailed findings about cell death processes will contribute to our understanding of human diseases of unknown aetiology, including autoimmune diseases. Judging from the rapid progress of this field so far, we may be able to translate our knowledge about cell death into new therapies for an increasing range of diseases in the near future.

Box 1 | **The future of research in the cell death field**

Key questions concerning the molecular mechanisms of cell death

- In apoptosis, caspase 3 cleaves more than 500 cellular proteins, of which only some have been related to apoptosis. It is not known whether the cleavage of other molecules represents bystander cleavage or whether these molecules have specific roles in the apoptotic programme.
- Flippases and scramblases have been shown to be involved in apoptotic phosphatidylserine (PtdSer) exposure. Elucidation of the molecular mechanism of how phospholipids carrying hydrophilic heads and hydrophobic tails are translocated between the two leaflets of plasma membranes is challenging.
- Cells that undergo apoptosis are swiftly engulfed. Whether the cells undergoing pyroptosis or necroptosis are engulfed or not is unknown. If these dead cells are engulfed, the molecular mechanisms involved need to be addressed.
- In *Caenorhabditis elegans*, specific nerve cells called 'linker cells' die with an apoptotic morphology but without showing caspase activation[101]. In addition, cells die by processes called 'ferroptosis', 'cornification', 'netosis' and 'autophagic cell death'; the detailed molecular mechanisms and physiological roles of these death processes are unknown and are being actively investigated.

Physiological and immunological issues

- The BIM and FAS apoptotic systems are involved in the process of lymphocyte selection. What activates the apoptosis programme during lymphocyte development is unknown.
- In tissue regeneration and embryogenesis, apoptotic cells produce mitogens and morphogens to compensate for the dead cells or to shape the growing embryo[102]. Whether these signals have roles in the immune system remains to be studied.
- Damage-associated molecular patterns (DAMPs) released from the cells that have undergone secondary necrosis activate the immune system and trigger autoimmune diseases in autoimmunity-prone mice. A detailed molecular understanding of how DAMP release triggers autoimmunity in this setting remains elusive.
- Necroptosis and pyroptosis are observed in cells that are infected by bacteria or by a virus. Whether these cell death processes occur under physiological conditions or during developmental processes remains to be studied.

Cell death in the context of tumour immunology

- A subset macrophages or dendritic cells that engulf apoptotic cells evokes antitumour activity[103]. How this happens is unknown.
- The US Food and Drug Administration (FDA) recently approved venetoclax (also known as Venclexta and ABT-199) — a B cell lymphoma 2 (BCL-2)-selective BCL-2 homology 3 (BH3) mimetic that activates the intrinsic apoptotic pathway[104] — for the treatment of patients with chronic lymphocytic leukaemia[105,106]. Whether other forms of cell death (such as the extrinsic pathway of apoptosis, necroptosis and pyroptosis) could be exploited for the treatment of patients with cancer remains to be studied.

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Competing interests statement
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