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STATE-OF-THE-ART REVIEW

Inflammatory cell death induced by cytotoxic lymphocytes: a dangerous but necessary liaison

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Cytotoxic lymphocytes (CLs), and more specifically Tc and NK cells, are the main executors of cell death in the immune system, playing a key role during both immunosurveillance and immunotherapy. These cells induce regulated cell death (RCD) by different mechanisms, being granular exocytosis and expression of death ligands the most prominent and best characterized ones. Apoptosis, a traditionally considered low-inflammatory type of cell death, has been accepted for years as the paradigm of RCD induced by CLs. However, several recent studies have demonstrated that NK cells and Tc cells can also induce more inflammatory forms of cell death, namely, necroptosis, pyroptosis, and ferroptosis. Activation of these highly inflammatory types of cell death appears to critically contribute to the activation of a successful antitumour immune response. Additionally, the role of specific cell death pathways in immunogenic cell death is still under intense debate, especially considering the interconnections with other inflammatory forms of cell death. These evidences, together with the advent of new cancer immunotherapies, highlight the necessity to deepen our understanding of the link between the cell death triggered by CLs and inflammation. This knowledge will be instrumental to maximize the antitumour potential of immunotherapies, minimizing deleterious effects associated with these treatments. In this review, we will briefly summarize the main features of apoptosis, necroptosis, pyroptosis and ferroptosis, to subsequently discuss the most recent evidences about the role of these RCD

Abbreviations
APAF-1, apoptotic protease-activating factor 1; BAK, BCL-2 antagonist/killer; BAX, BCL-2-associated X protein; CARs, chimeric antigen receptors; CLs, cytotoxic lymphocytes; CRS, cytokine release syndrome; DAI, DNA-dependent activator of IRFs; DAMPS, danger-associated molecular patterns; DD, death domain; DISC, death-inducing signalling complex; E3, ubiquitin ligase; ER, endoplasmic reticulum; FADD, Fas-associated death domain; GSD, gasdermin; GZM, granzyme; HMGB1, high-mobility group box 1 protein; ICAD, Inhibitor of caspase-activated DNase; IFN, interferon(s); ITAMs, immunoreceptor tyrosine-based activating motifs; ITIMs, immunoreceptor tyrosine-based inhibitory motifs; MAPKs, mitogen-activated protein kinase(s); MHC, major histocompatibility complex; MLKL, mixed lineage kinase domain like pseudokinase; MOMP, mitochondrial outer membrane permeabilization; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, natural killer; PAMPS, pathogen-associated molecular patterns; PRF, perforin; PRRs, pattern recognition receptor(s); PUFAs, polyunsaturated fatty acids; RCD, regulated cell death; RHIM, RIP-homotypic interacting motif; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; ROS, reactive oxygen species; Tc, cytotoxic T cells; TCR, T-cell antigen receptor; TILs, infiltrating lymphocytes; TLR, Toll-like receptor; TNF, tumour necrosis factor; TNFR1, TNF-receptor 1; TNFR1-SC, TNFR1-signalling complex; TNFSF, TNF-superfamily; TRAIL, TNF-related apoptosis inducing ligand; ZBP1, Z-DNA binding protein 1.

pathways during the elimination of cancer cells mediated by CLs and its modulation to increase the efficacy of cancer immunotherapy.

Introduction

Cytotoxic lymphocytes (CLs), including natural killer (NK) and cytotoxic T (Tc; $CD8^+\alpha\beta T$ and subsets of $CD4^+\alpha\beta T$, NKT and $CD8^{+/-}\gamma\delta T$) cells are the main executors of cell death in the immune system, playing a key role in the elimination of infected or transformed cells [1–4]. Tc and NK cells recognize target cells by different mechanisms in a complementary way to maximize elimination of affected cells, overcoming potential immune-evasion strategies of pathogens and tumours. Tc cells use their T-cell antigen receptor (TCR) to recognize and eliminate target cells that present foreign immunodominant peptides bound to MHC (Major Histocompatibility Complex) molecules (class I or II for $CD8^+$ T or $CD4^+$ T cells, respectively) expressed on the surface of target cells [5]. Thus, Tc cell-mediate elimination of transformed or infected cells is triggered upon recognition of specific antigens. In contrast, NK cell activation is not regulated by antigen expression, but rather by detection of cellular stress [6]. To this aim, NK cells present a complex system of inhibitory and activating receptors specialized at detecting reduction in MHC expression and stress in target cells, respectively, due to infection or transformation [7]. Activating receptors provide NK cells with a strong stimulus throughout tyrosine-based activating motifs (ITAMs), whilst inhibitory receptors contain tyrosine-based inhibitory motifs (ITIMs) within their cytosolic tail. The balance between activating and inhibitory signals determines the activation of NK cells, providing signals for NK cells to become activated and display cellular cytotoxicity. Thus, NK cells can eliminate target cells that are not recognized by Tc cells because either have downregulated MHC expression and/or do not express foreign antigens but present increased stress signals.

Both NK and Tc cells share a wide molecular arsenal capable of inducing cell death upon activation and recognition of target cells. The main mechanisms used to trigger cell death are the granular exocytosis pathway, mainly involving the perforin/granzyme (PRF/GZM) system, and the expression and release of death ligands [8]. Both systems were originally described to induce mainly apoptosis in the target cells, although it has been recently described that they can also induce other types of RCD such as necroptosis and pyroptosis. These findings open a new perspective on the consequences of cell death induced by CLs for the activation of inflammatory pathways, which can lead to the regulation of second immune responses that might help to fight cancer and infection. In this line, recent evidences showed that the activation of specific cell death pathways by traditional anticancer therapies (radiotherapy and some chemotherapies) can induce the release of danger inflammatory signals, which can trigger the activation of antigen-restricted specific immune response against cancer cells, a process known as immunogenic cancer cell death, which helps to eliminate tumours and might provide immunological memory against recurrent cancer cells. Indeed, the most recent evidences show that CLs are able to induce cancer immunogenic cell death, inducing tumour antigen spread and protection against secondary tumours [9–11]. Thus, all these recent studies suggest that a better understanding of the link between the novel forms of cell death activated by CLs and the activation of host immunity against endogenous tumour antigens might provide new opportunities to extend the efficacy of cancer immunotherapy to refractory cancer types, the so-called cold tumours, by promoting appropriated antitumoural inflammatory microenvironments. In this review, we will present the main mechanisms of regulated cell death, highlighting the interconnection between them, as a preface to discuss the potential role of these forms of cell death and the most novel pathways regulating them in the elimination of cancer cell by CLs.

Mechanisms of regulated cell death

Over the last years, the cell death field has witnessed an important revolution, with the demonstration of how different types of RCD, mainly apoptosis, necroptosis, and pyroptosis, that were originally regarded as independent entities, are indeed molecularly interlinked, and how those different interconnected cell death pathways are critically involved in the development and treatment of different pathologies-like cancer, infectious and inflammatory/autoimmune diseases, ageing or degenerative diseases among others. Whilst apoptosis has traditionally been considered to be immunologically silent due to the fact that no intracellular content is released, necroptosis, pyroptosis and ferroptosis present the common feature of disrupting

the plasma membrane, resulting in immunogenic cell death. Yet, the potential role of apoptosis in immunogenic cell death is not clearly understood, since it has been described that apoptotic cell death induced by radiotherapy and specific chemotherapy agents could be immunogenic [12,13].

Apoptosis

Since the definition of Apoptosis by Kerr in 1972 [14], apoptotic cell death has been classically regarded as the gold standard modality of regulated cell death (RCD) as opposed to Necrosis, which was considered to happen in response to external physico-chemical insults [15]. Technically, the term Apoptosis was coined to classify the type of cell death according to morphological aspects, such as rounding-up of the cell, reduction of cellular volume, chromatin condensation, nuclear fragmentation, plasma membrane blebbing and engulfment by resident phagocytes. However, the discovery of Cysteine–ASPartic proteASES (Caspases) [16] as the main executioners of apoptosis, and the advent of synthetics caspase inhibitors such as Z-VAD-fmk, QVD or Emricasan widened the definition of Apoptosis towards 'caspase-dependent cell death', which can be fully inhibited by the use of caspase inhibitors [17]. A key feature of apoptosis is that the cell membrane maintains its integrity until the final stages of the process, avoiding release of intracellular content to the extracellular compartment and preventing inflammation [14]. One of the main biologic roles of apoptosis is to eliminate cells that are superfluous or that are irreparably damaged, playing a key role during development and homeostasis of tissues throughout adult life. Importantly, evasion of apoptosis is considered as one of the so-called 'Hallmarks of cancer' [18] and can play an important role in the development of chemoresistance to conventional therapeutic drugs [19].

Mechanistically, apoptosis can be activated by two different pathways: the extrinsic pathway and the intrinsic pathway [20]. The intrinsic pathway is also known as mitochondrial apoptotic pathway due to the key role mitochondria plays as its central regulator, although other organelles-like ER or lysosomes might also regulate this process. This pathway is tightly regulated by members of the Bcl-2 superfamily, which includes both, pro-apoptotic and anti-apoptotic components. The delicate regulation of the balance between pro-and anti-apoptotic proteins determines the decision between life and death [21]. One of the main physiological events that activate the intrinsic pathway is cytokine or growth factor deprivation, which favours the activity of the pro-apoptotic members of the Bcl-2 family, either by phosphorylation-driven activation or by up-regulating their expression [22]. Alternatively, the intrinsic apoptotic pathway can be also triggered by genotoxic damage (a process exploited by chemotherapy or radiotherapy), by damage to cellular organelles or by signalling platforms (ER stress), mitochondrial damage or excessive mitogenic stimulation [20]. For example, DNA damage induces p53 activation to drive transcription of PUMA and NOXA, two BH3-only pro-apoptotic proteins that activate the pro-apoptotic pore-forming proteins BCL-2-associated X protein (BAX), and/or BCL-2 antagonist/killer (BAK) at the mitochondrial surface enabling them to oligomerize and form macropores in this membrane. These pores cause mitochondrial outer membrane permeabilization (MOMP), resulting in the release of apoptogenic proteins from the intermembrane space as cytochrome c, which with the scaffold protein apoptotic protease-activating factor 1 (APAF1) and pro-caspase 9 form the apoptosome [23].

The extrinsic apoptotic pathway is triggered by the interaction of death ligands with their cognate death receptors on the surface of target cells. Death ligands are a subgroup of the TNF-superfamily and include TNFα, FasL/CD95L and Apo2L/TRAIL [24]. These ligands are mainly produced by immune cells although other cells have also been found to express them under different circumstances. Among them, FasL/CD95L and Apo2L/TRAIL are prominently apoptotic, whilst TNF only triggers apoptosis under certain conditions [25]. The different death ligands present certain differences in their signalling cascades, although they all induce the activation of caspase 8 at multi-protein signalling complexes (referred to as DISC for FasL and TRAIL and as Complex II for TNF) in a FADD-dependent manner [26,27]. Both DISC and Complex II also recruit other important accessory proteins acting as regulatory hubs of the signalling cascade such as cFLIP and RIPK1 [27]. Noteworthy, despite the eminent pro-cell death role of these complexes, they can also activate noncell death prosurvival and/or proliferative pathways such as NF-kB and MAPKs, which are also involved in the expression of pro-inflammatory cytokines [28–31]. These findings unveil an intricated biology, with a single signalling molecule being able to trigger different signalling pathways with consequences that may have a direct impact on the inflammatory outcome of the cell death triggered by Death Ligands.

As indicated above, apoptosis is characterized by the formation of apoptotic bodies that retain the intracellular content, which express specific signals to be found and endocyted by phagocytic cells. However,

most recent evidences suggest that apoptotic cells killed under specific circumstances might release danger signals which would promote inflammation and the activation of host immunity against the dying cells [12,32]. This form of apoptosis somehow resembles lytic forms of cell death, in which membrane permeability is disrupted and intracellular danger signals with inflammatory potential are released to the extracellular space similarly to necroptosis or pyroptosis. Here it should be indicated that some studies have found release of danger signals by apoptotic cells such as Calreticulin (associated with outer part of the plasma membrane), HMGB1 or cytokines like members of the IL-1 family and type I interferons (IFNs) [33,34]. More studies are required to clearly address whether the release of these signals happens before, during, or after cell membrane is permeabilized. In addition, it should be analysed whether the release of danger inflammatory signals is a consequence of cell death or it occurs in parallel to cell death. Here a potential hypothesis could be that tumour cells exposed to the apoptotic stimulus releases inflammatory cytokines and other danger signals-like type I IFN before they die due to the activation of cellular stress. In this scenario, apoptotic cells in the presence of type I IFN produced by cells before they die, would induce the activation of the host immune response, leading to immunogenic cell death [34,35]

Necroptosis

Caspase-independent forms of RCD have been known for decades [36,37], although due to lack of knowledge on their mechanistic details they were mostly regarded as 'caspase-independent apoptotic cell death' [38]. Some seminal studies described what appeared to be a certain type of regulated necrotic cell death, only occurring upon caspase inhibition [39–41]. Morphologically, this cell death was very different from apoptosis, presenting cytoplasmic swelling and rupture of plasma membrane, and involving a direct pro-inflammatory effect by the release of intracellular contents into the extracellular compartment. Further studies shed light on the mechanism behind this caspase-independent form of cell death, finding that rupture of the plasma membrane was caused by the formation of large pores mediated by the oligomerization of the pseudo-kinase MLKL [42–45]. This oligomerization is triggered by a direct phosphorylation of MLKL by activated RIPK3 [46], which in turn can be activated by RIPK1 [47,48] or ZBP1/DAI [49]. Necroptosis has been best characterized in the context of TNF signalling. TNF stimulation triggers the formation of the TNFR1-signalling complex (TNFR1-SC) or Complex I [25]. Within this complex, RIPK1 is ubiquitinated and phosphorylated in several sites by different E3 ligases and kinases, respectively [50,51], serving as inhibitory checkpoints that prevent the activation of the kinase activity of RIPK1. However, under certain conditions, a deficient or incomplete activity of the Complex I inhibitory checkpoints triggers the activation of RIPK1, leading to the formation of the secondary Complex II [52–56]. This complex, mainly formed by RIPK1, FADD and caspase 8, has the potential to activate both apoptosis and necroptosis depending on the cellular context. Under conditions of caspase inhibition, the Complex II evolves towards the Necrosome by the recruitment of further RIPK1 and RIPK3 molecules, forming characteristic amyloid-like structures mediated by homotypic RHIM-RHIM interactions [57], ultimately resulting in the activation of MLKL. This RIPK1-mediated necroptosis can be blocked by direct inhibition of the kinase activity of RIPK1 by Necrostatins [58,59].

Altogether, necroptosis is regulated by RHIM domain-containing proteins, such as the aforementioned RIPK1, RIPK3 and ZBP1/DAI, and also TRIF, an adaptor protein involved in TLR3 and TLR4 signalling which enables the induction of necroptotic cell death by activated Pattern Recognition Receptors (PRRs) [60]. Importantly, being a lytic form of cell death, necroptosis induces the release of intracellular content to the extracellular space, including DAMPS (Danger Associated Molecular Patterns) such as ATP and HMGB1, which can trigger a strong pro-inflammatory response [61].

Pyroptosis

Pyroptosis is a lytic form of RCD, initially defined by the activation of caspase 1 linked to the release of IL-1β [62]. However, more recent evidences show that Pyroptosis is also characterized by being executed by members of the gasdermin (GSDM) family upon activation of pro-inflammatory caspases at the inflammasome [63–65] (Fig. 1). The inflammasome is a multimeric complex, formed upon sensing of pathogen-derived or host-derived danger signals [66,67]. Activation of the canonical and noncanonical inflammasomes leads to the recruitment and activation of the inflammatory caspases 1 and 4, respectively, in humans (or caspases 1 and 11 in mice). Once activated, these caspases cleave members of the GSDM family at the so-called central linker region, being GSDMD and GSDME/DFNA5 the best characterized so far [63,64]. Cleavage of GSDMs induces the release of a N

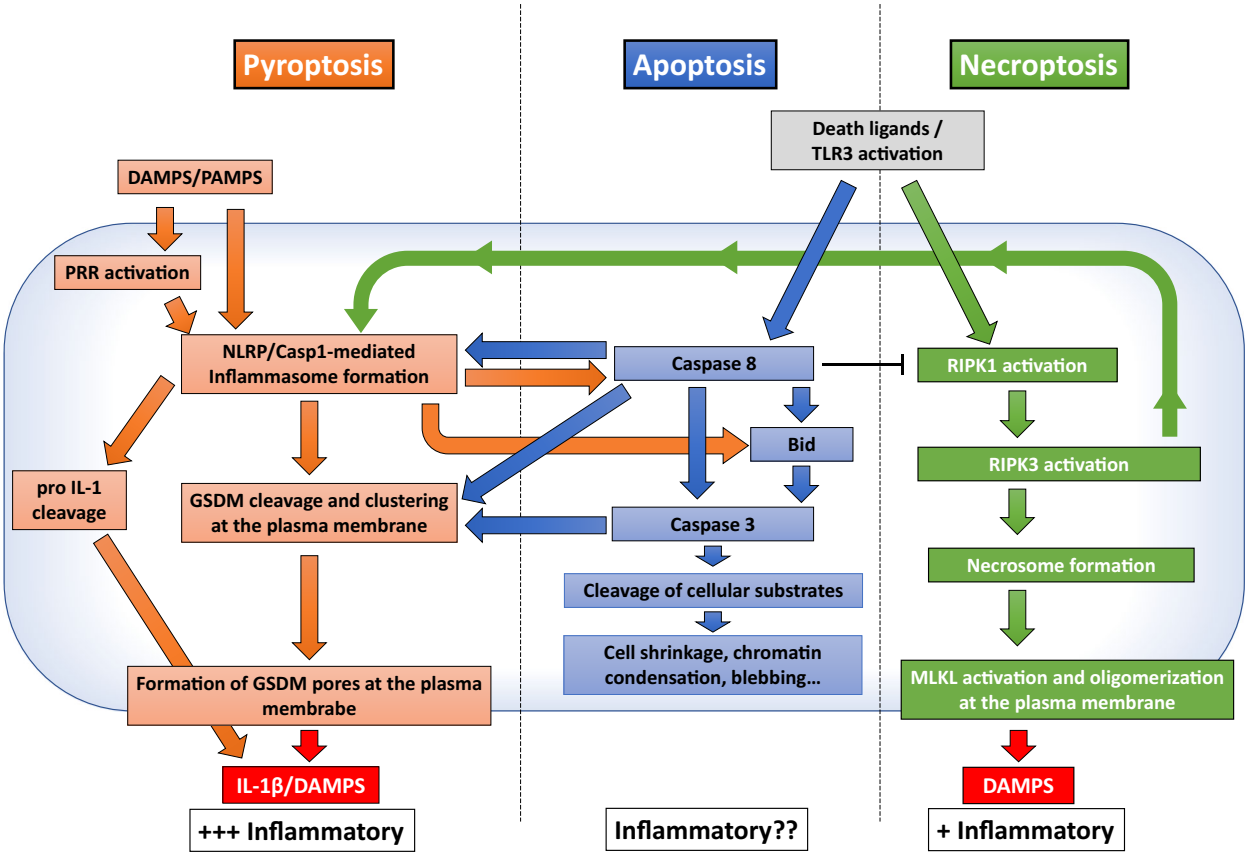

Fig. 1. Overview of the main interconnections between Apoptosis, Necroptosis and Pyroptosis. Apoptosis, necroptosis and pyroptosis are interconnected by a complex molecular network. Upon activation of Apoptosis, active caspase 8 quickly cleaves and inactivates RIPK1, effectively blocking the initiation of necroptosis. On the other hand, caspase-8 has been described to participate at the activation and formation of the NLRP3 inflammasome, either by playing catalytic or scaffolding roles depending on the cellular context. Similarly, RIPK3 has also been described to be involved in the formation of the inflammasome in some cell types. Inversely, caspase-1 activated during Pyroptosis has been described to cleave Bid, releasing its active processed fragment tBid, and linking activation of the mitochondrial apoptotic pathway with Pyroptosis. Finally, some members of the GSDM family can be processed by active caspases-8 and caspases-3 without the need for the formation of the inflammasome, producing an inflammatory type of cell death secondary to Apoptosis induction.

terminal fragment with capacity to oligomerize at the plasma membrane, forming pores and therefore compromising the integrity of the plasma membrane which causes a severe osmotic shock leading to a quick release of cytosolic content and necrosis [63]. A unique feature of Pyroptosis is the release of IL-1β and IL-18, mature forms of proIL-1 which result from the cleavage of proIL-1 by activated caspases-1 and/or caspases-4 after inflammasome formation [66,67]. Thus, Pyroptosis encompasses the processing of proIL-1 with activation of GSDMs, resulting in a particularly inflammatory type of cell death. This pathway has been classically connected to cell death activated in macrophages and epithelial cells as a host mechanism of protection against intracellular bacterial infections [62]. However, it has also been recently found that different cell types, including cancer cells, can undergo pyroptosis during treatment with radiotherapy or chemotherapy among others [68,69].

Ferroptosis

Ferroptosis is a recently described form of regulated necrosis [70], characterized by a lethal damage of the plasma membrane triggered by an increased peroxidation of polyunsaturated fatty acids (PUFAs). This peroxidation is caused by the interaction of free iron atoms with ROS during the so-called Fenton reaction [71], ultimately leading to the peroxidation of PUFAs. This peroxidation triggers a chain reaction, leading to more oxidized lipids, which can only be countered by the cellular lipid peroxidase, GPX4 [72]. Importantly,

GPX4 requires a constant regeneration mediated by glutathione, which in turn requires NADPH. *De novo* synthesis of glutathione requires cysteine, which is internalized by the glutamate–cysteine antiporter system X_C^-. Thus, defects in levels of functional GPX4 caused by cysteine deprivation, inhibition of the system X_C^- transporter or reductions of NADPH facilitate lipid peroxidation in cells susceptible to undergo lipid peroxidation. On the contrary, ferroptosis can be inhibited by iron chelators or reduction of PUFA levels either by inhibition of their synthesis or by scavenging of PUFAs and/or ROS [73].

Interconnection between the different types of RCD

Initially considered as independent processes, recent studies have described molecular connections between apoptosis, necroptosis and pyroptosis, in a way that several key proteins such as RIPK1, RIPK3 or caspase 8 act as molecular hubs for the regulation of the different cell death programmes, either by promoting or inhibiting specific pathways upon certain conditions [74] (Fig. 1).

One of the best characterized interconnections between RCD pathways is the one between apoptosis and necroptosis, mainly exerted by RIPK1 and caspase 8. As aforementioned, RIPK1 is a core component of the Complex II, which formation is directly inhibited by specific ubiquitination and phosphorylation events on RIPK1 serving as inhibitory checkpoints [50,51]. Therefore, RIPK1 acts as a regulatory hub for both apoptosis and necroptosis, especially in TNF signalling [75–77]. On the other hand, active caspase 8 quickly cleaves and inactivates RIPK1, effectively blocking the initiation of necroptosis [78,79]. Importantly, this cleavage can also be carried out by the hetero-dimer of caspase 8 and cFLIP, not being necessary a complete activation of caspase 8 [28,80]. Thus, even in conditions of relatively high cFLIP expression, which would prevent apoptosis, RIPK1 will still be cleaved and necroptosis inhibited. Only when caspase activity would be completely blunted, like in the presence of viral caspase inhibitors, necroptosis would proceed. Thus, once Complex II is fully formed, the degree of activation of caspase 8 is a key milestone ultimately controlling whether apoptosis or necroptosis will be initiated.

On the other hand, it is intriguing how members of the caspase family are the key mediators of two apparently contradictory modalities of RCD: the respectively low and high inflammatory forms of cell death apoptosis and pyroptosis, with caspases 3 and 1 being, respectively, the most relevant mediators of both pathways. Nevertheless, over the last few years several studies have unveiled a certain cross-reactivity between pro-apoptotic and pro-inflammatory caspases, suggesting that the traditional caspase classification as apoptotic or inflammatory members should be carefully considered. In this regard, as discussed above, activation of caspase 1 at the inflammasome induces cleavage of proIL-1 rendering the active mature forms IL-1β and IL-18 and on the other hand cleaves and activates members of the GSDM family which execute pyroptosis. However, caspase 1 has also been reported to be able to induce apoptosis. Thus, in cells lacking GSDMD, caspase 1 was shown to trigger Bid cleavage followed by activation of caspases 3 and 7 [81,82]. Inversely, caspase 8 has been described to be recruited to and activated at the inflammasome under certain conditions [83–86]. This inflammasome-activated caspase 8 was shown to directly regulate IL-1β and inflammation or even to trigger an apoptosis-like cell death [87] in cells lacking caspases1/11. On the other hand, several studies showed a caspase 8-dependent activation of the NLRP3 inflammasome following apoptosis [88–92]. Nevertheless, the activation of the inflammasome in these scenarios would not directly involve caspase 8 as a core component, but it would rather occur secondarily to the activation of apoptosis, therefore requiring the catalytic activity of caspase 8. However, two very recent independent studies have demonstrated that caspase 8 plays a key role as scaffold for the formation of the inflammasome, independently of its protease activity [83,84]. In both studies, the authors found that mice expressing a catalytically inactive mutant of caspase 8 caused a necroptosis-independent death during embryonic development, which was due to an uncontrolled formation of the inflammasome. Thus, caspase 8 would be directly involved not only in the cleavage of substrates but also in the scaffolding of the inflammasome complex. Similarly, RIPK3 has also been described to be involved in the formation of the inflammasome in some cell types upon TLR activation [90]. All these novel pathways of inflammasome activation would lead to the release of pro-inflammatory members of the IL-1 family promoting the generation of a potentially protective inflammatory microenvironment. Whether this process is protumoural, due to activation of cell transformation pathways, or antitumoural due to activation of host immunity should be carefully considered and analysed in every specific model.

Very recently, several reports have demonstrated that caspases 8 and 3 activated during canonical apoptotic stimuli can directly cleave and activate GSDMD and GSDME/DFNA5 respectively, bypassing the need

for the inflammasome [93–95]. These studies clearly illustrate a direct link between apoptosis and pyroptosis, which functionally results in the addition of an inflammatory response to low-inflammatory pathways of cell death.

Last but not least, a connection between receptors traditionally thought to be exclusively involved in the regulation of immune cell activation (like PRRs) and different forms of RCD has been recently found. In this context, abnormal levels of DNA and RNA fragments released during cell death are accumulated into the cytosol as secondary by-products. These nucleic acids are then sensed by innate nucleic acid sensors such as cGAS-STING, ZBP1/DAI, caspase 1, TLR3 or members of the NLRP family [96], which simultaneously trigger the expression of pro-inflammatory cytokines mediated by NF-kB or the activation of IFN responses resulting in the secretion of type I interferons. In addition, these innate nucleic acid sensors can also activate RCD pathways that might be important to control infection by intracellular pathogens or during tumoural transformation. Altogether, sensing of endogenous nucleic acids by innate PRRs results in a secondary loop which amplifies the pro-inflammatory signature by the release of cytokines and type I IFN, and triggers additional RCD pathways, potentiating the immunogenic response [96]. However, there are still many questions that remain unanswered regarding the regulation of these innate nucleic acid sensors and the physiological relevance of these mechanisms.

In summary, all these studies are unveiling a complex molecular network controlling that cell death takes place in an orchestrated manner and in a hierarchical form. Thus, data so far suggest that upon infection with a pathogen (either viral or bacterial), the host defence mechanisms would firstly activate caspase 8-dependent apoptosis. In this scenario, a successful and complete activation of caspase 8 would also inhibit the more inflammatory necroptosis and pyroptosis, as a safe means to eliminate pathogen-infected cells without triggering a strong inflammatory response in the surrounding tissue. However, in situations in which pathogens have developed ways to block this first line of defence and promote host cell survival to ensure pathogen replication, necroptosis and/or pyroptosis would be activated depending on the cellular context and the cell type in each case, triggering a more inflammatory and immunogenic second line of defence. This co-dependent regulation would act as a safeguard mechanism, developed by pluricellular organisms as a product of millions of years of co-evolution with pathogens that evolved to evade cell death mechanisms. Similarly, this mechanism developed by pathogen-mediated evolutionary pressure would work in an analogous way in the case of cancer cells that had learnt to avoid the intrinsic apoptotic mechanisms activated during cell transformation.

Cytotoxic mechanisms exerted by NK and Tc cells

A central role of both innate and adaptive immunity is the ability to eliminate infected and transformed cells. This role is mainly exerted by NK and different Tc cell subsets as indicated in the introduction [1–4]. During the last 30 years, one of the main aims in cancer research has been to try to exploit the natural ability of these immune cells to recognize and eliminate cancer cells. Thanks to the greater understanding of cancer immunity accumulated in this period, this objective has seen the light in recent years in the form of new immunotherapies with great success, although still limited to some types of cancer. Among these treatments, the development of engineered T and NK cells including chimeric antigen-receptors (CARs) and transgenic TCRs or the transfer of *in vitro* expanded TILs or NK cells as well as the use of recombinant antibodies have revolutionized cancer therapy [97–99].

NK and Tc cells present fundamental differences in how they get activated and recognize cancer cells, although they share the basic mechanisms to induce cell death on target cells: granular exocytosis and release of death ligands [8]. The PRF/GZM system (also termed granular exocytosis) consists on the release of preformed cytotoxic granules [100–102], which is a multi-step regulated process, triggered by the formation of an immunological synapse between the effector and the target cell. Within this synapse, several receptors located on the surface of the effector cells can interact with their respective ligands on the target cell, resulting in a reorganization of the actin cytoskeleton and a subsequent mobilization of the preformed cytosolic granules towards the synapse, where they fuse with the plasma membrane, releasing their content into the synapse space [100]. Among other components, the cytotoxic granules contain the pore-forming protein perforin (PRF) and several serine-proteases called granzymes (GZMs). In addition, human granules contain granulysin, a membrane-interacting protein with antimicrobial activity, albeit a direct cytotoxic antitumoural function is not clear yet [103]. To date, five human granzymes (ten in mice) are known: GZMA, GZMB, GZMH, GZMK and GZMM. Although they all share a high sequence homology, they differ in their substrate specificity and their physiological functions [104–106]. Thus, once released into

the synapse, PRF monomers insert into the target cell membrane, polymerizing and forming pores which facilitate the intracellular delivery of GZMs. The cytotoxic potential of the different GZMs is still not clear [107]. Of all GZMs, most independent studies agree that GZMB is the member with a highest cytotoxic potential in both mouse and human CLs. Once delivered into the target cell, GZMB can initiate apoptosis by cleaving several substrates such as the BH3-only protein Bid, Mcl-1 or caspase 3. Alternatively, GZMB can also induce caspase-independent cell death by cleaving other cell substrates such as tubulin or ICAD leading to cell destruction [8,108]. In contrast, the cytotoxic potential of other GZMs, such as GZMA, is still unclear and might be influenced by the species and by the target cell. In addition, other biological functions of GZMs have been described in recent years, including direct inactivation of intracellular pathogens or regulation of different biological responses at the extracellular space including inflammation, ageing or cardiovascular damage [109–111].

The so-called death ligands are a subset of the TNF-superfamily proteins (TNFSF) whose receptors (death receptors) contain a cytosolic death domain (DD), which allows them to induce cell death pathways upon activation [24]. Death ligands are mainly expressed by immune cells like activated macrophages, $CD4^+$ T cells, $CD8^+$ T cells and NK cells. They are expressed as transmembrane proteins, attached to the extracellular membrane or to the surface of exosomes, which are released upon activation. Despite their name, death receptors can also induce signalling pathways independent of cell death, such as activation of NF-kB or MAPKs, which result in the expression of different genes [28–31]. Of note, although TNF is technically considered as a death ligand, its main signalling outcome is to induce the aforementioned non-cell death pathways, only inducing cell death under certain circumstances.

Finally, another major effector mechanism of CLs is the expression and release of IFNγ. This cytokine is mostly secreted by activated T cells (including CD4-Th1, CD8 and γδT cells) and NK cells and plays a key role in antiviral response [112]. IFNγ is a pleiotropic cytokine, inducing the expression of hundreds of genes in target cells, most of them implicated in inflammatory signalling, cell cycle regulation and expression of transcriptional activators. In addition, IFNγ also modifies the expression of several genes involved in the regulation of different RCD pathways such as Bcl2-family proteins, caspases, RIPK3 or death receptors, greatly sensitizing target cells to cytotoxicity induced by CLs [113–118]. More recently, IFNγ has been also described to directly trigger cell death by different means when combined with other inflammatory stimulus [119–121]. However, direct cytotoxicity exerted by IFNγ is still controversial, and it could be explained by the deep expression reprogramming of genes involved in cell death within target cells (including the simultaneous up-regulation of death receptors and autocrine expression of death ligands). In this context, IFNγ stimulation would induce a secondary, death-receptor-mediated cell death. In any case, IFNγ plays a key role at modulating and modifying the sensitivity of target cells to different RCD pathways, definitively impacting the cytotoxic ability of CLs.

The contribution of each pathway (granule exocytosis and death ligands) to the elimination of cancer cells by CLs is still not completely understood and might depend on the type of effector cells. For example, recent evidences suggest that the role of FasL in chronic retrovirus infection might be more relevant for cytotoxic $CD4^+$ T cells than for $CD8^+$ T cells [122]. In addition, the type of target cell, especially the expression of intracellular inhibitors and/or mutations affecting to cell death pathways, is also likely affecting the cytotoxic potential of PRF/GZMs and, more likely that of death ligands. Albeit this is a very interesting and relevant topic in the field of cancer immunity and immunotherapy, we will not discuss it here. Recent reviews on this topic have been published [8,123].

Caspase-independent cell death and cytotoxic lymphocytes

Although apoptosis has been considered the paradigm of cell death pathway activated by CLs, in line with the most recent advances in the cell death field, a role for other cell death pathways (including the crosstalk between them) has also been recently described during the elimination of cancer cells by NK and Tc cells (Fig. 2). Since apoptosis induced by CLs has been the topic of several excellent reviews, here we will focus on the 'novel' cell death pathways activated by CLs. Previous evidences demonstrated that CD8+ Tc and NK cells were still able to eliminate target cells even in the presence of apoptosis inhibitors, both *in vitro* and *in vivo*, using the granule exocytosis pathway [11,108,114–117]. This strongly suggested that, besides apoptosis, CLs could activate alternative forms of cell death. More recently, it has been confirmed that CLs are able to induce caspase-independent forms of cell death, namely, necroptosis, pyroptosis and ferroptosis, which will most likely mechanistically explain the former studies. However, this is an emerging field, and more studies are required to confirm these results and

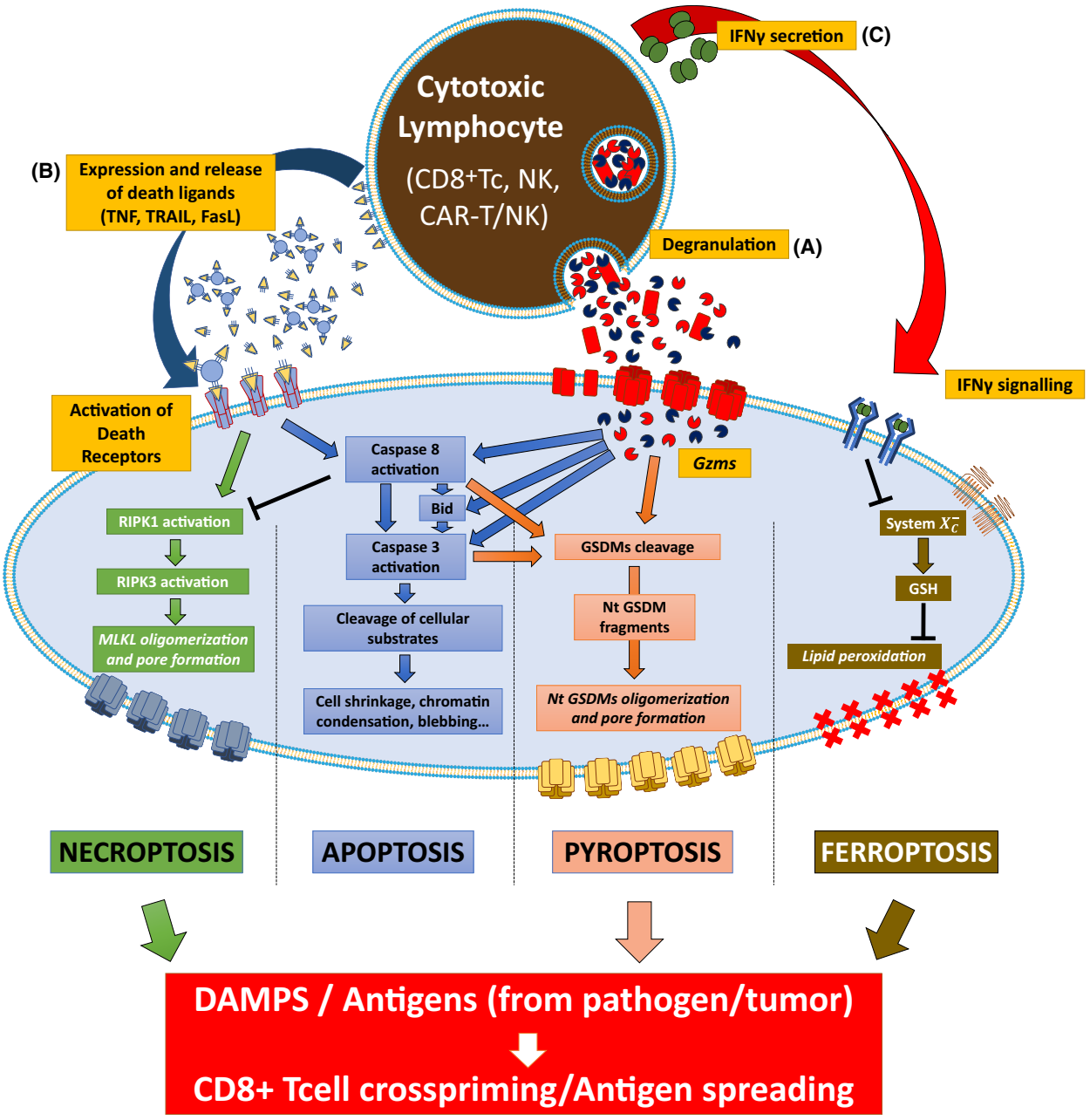

Fig. 2. Overview of the different cell death pathways triggered by cytotoxic lymphocytes. Upon activation, cytotoxic lymphocytes can induce killing of the target cell by different means, namely, by degranulation (PRF/GZM system), by expression and release of death ligands and by secretion of IFNγ. During degranulation (A), PRF induces the formation of pores in the cell membrane of target cells which facilitate the intracellular delivery of GZMs. Once in the cytosol, GZMs can trigger the activation of different RCD pathways by cleaving different substrates. Thus, GZMB induces Apoptosis by cleaving substrates as Bid, Mcl-1 or caspases-8 and caspases-3. In addition, GZMB can also induce pyroptosis by directly cleaving GSDME, whilst GZMA can also cleave and activate GSDMB. Death ligands (B) induce Apoptosis by activation of the extrinsic apoptotic pathway through activation of caspase-8, but they can also induce necroptosis depending on the cellular context. Irrespectively of the activating mechanism, active caspases-8 and/or caspases-3 can directly cleave and activate GSDMD and GSDME, respectively, linking apoptosis and pyroptosis. Finally, IFNγ (C) modifies the expression of several genes involved in the regulation of different RCD pathways, greatly sensitizing target cells to cytotoxicity induced by CLs. In addition, IFNγ has been described to induce a down-regulation of the system X_C^-, impairing the uptake of cysteine and therefore enabling lipid peroxidation and activation of ferroptosis. The relative contribution of each of these RCD pathways to the cytotoxicity of cytotoxic lymphocytes may depend on different variables

such as the activation status of the effector cells or the cellular context of the target cells. In any case, depending on the predominant resulting type of cell death, dying cells may release potent pro-inflammatory factors such as DAMPS or IL-1β together with intracellular antigens, which would result in a pro-inflammatory environment, enhancing CD8+ T-cell crosspriming and antigen spreading.

to understand their physiological relevance as discussed below.

Necroptosis

The main physiological role of necroptosis appears to be the control of infection during the innate immune response. Considering caspase-8 inactivation is a prerequisite to activate necroptosis and together with its pro-inflammatory nature (in contrast with the non-inflammatory one of apoptosis), necroptosis is currently viewed as a sort of backup mechanism for apoptosis, triggered in scenarios of pathogen-mediated caspase inhibition and encompassing a robust pro-inflammatory component to alert the immune system. Besides this, a role for necroptosis at controlling T-cell homeostasis has been described in certain situations. Thus, in mice lacking caspase-8 or FADD expression, an impaired expansion of T cells following TCR activation was observed [124–126]. This accumulation of T cells could be restored by using a kinase inhibitor of RIPK1 or by genetic co-deletion of RIPK1 or RIPK3 [126–128]. On the other hand, necroptosis has been implicated in the elimination of excessive T cells during the contraction phase of a viral infection, which could be reverted by co-deletion of RIPK3 [128]. However, to date no convincing evidences of the physiological contribution of necroptosis to CL-induced cell death during tumour immunosurveillance or immunotherapy have been reported. Nevertheless, some studies have demonstrated necroptosis can be potentially implicated in the activation of the adaptive immune response. In this regard, Yatim *et al.* [129] elegantly demonstrated that necroptotic cell death (induced by stimuli other than CLs) was able to produce a successful crosspriming of Tc cells against antigens present in the dead cells. In this study, the authors found that cells dying by necroptosis induced dendritic cell maturation, which in turn activated $CD8^+$ Tc responses. Interestingly, the authors also found that necroptotic cells were able to simultaneously activate NF-kB in a RIPK1-dependent manner, which was in turn responsible for the expression of functional pro-inflammatory cytokines such as IL6. Moreover, release of DAMPS was not sufficient to initiate $CD8^+$ Tc cell responses on its own, but the simultaneous RIPK1-mediated NF-kB activation was also required. On the other hand, Aaes *et al.* [130] also found that necroptotic cells were able to induce maturation of dendritic cells and activation of $CD8^+$ Tc cells in a different model, confirming the relevance of necroptotic cell death in crosspriming adaptive T-cell responses. However, and in contrast to the study by Yatim *et al.*, in this study the authors found that the immune activation was independent of the NF-kB pathway, only relying on DAMP release. This difference might be due to experimental technicalities, although fundamental biological differences between the models used cannot be excluded. At present, the activation of necroptosis by CLs during cancer immunosurveillance and immunotherapy is still not clear. This topic deserves more research, as harnessing the intrinsic immunogenic nature of this type of cell death could be exploited for improving current cancer immunotherapy strategies.

Ferroptosis

$CD8^+$ Tc cells activated during treatment with an immune checkpoint inhibitor were recently described to induce ferroptosis in tumour cells, functionally contributing to the antitumour effect of Tc cells [131]. In this study, the authors found that treatment of mice bearing ovarian tumours with the checkpoint inhibitor anti-PDL1 resulted in an increase of lipid peroxidation within tumour cells, leading to ferroptosis. This peroxidation was apparently mediated by IFNγ secreted by activated Tc cells, which induced a down-regulation of components of the glutamate–cysteine antiporter system X_C^-, impairing the uptake of cysteine and therefore enabling lipid peroxidation and activation of ferroptosis. However, this study did not clarify the contribution of granule exocytosis and death ligands to this process, which would be expected to activate apoptosis, and, thus, it would be very interesting to reveal the relative contribution of apoptosis and ferroptosis during the elimination of cancer cells by CLs. Although this finding is still pending of validation by independent studies, it will be interesting to analyse if ferroptosis contributes to the immunogenicity of cell death induced by Tc cells recently found [9,10]. Specially, since it was recently shown the immunogenic potential of ferroptotic cell death induced by accumulation of intracellular iron ions mediated by Ras activation [132].

Pyroptosis

Three very recent studies have demonstrated that granzymes released by different types of activated CLs can trigger pyroptosis either by direct cleavage of GSDMs or through the activation of caspase 3. In the first publication, Liu *et al.* [133] described how caspase 3 activated by GZMB released by CAR T cells cleaved and activated GSDME, triggering pyroptotic cell death in target cells. Importantly, the authors also found that co-culture of supernatants obtained from this pyroptotic cell death with macrophages induced a secondary release of IL-1β and IL-6, two important markers of cytokine release syndrome (CRS). In this line, they confirmed that pyroptosis triggered CRS in CART cell treated mice, and they linked these results with CRS observed in some patients treated with CD19-CAR T-cell therapy, correlating the relative expression of GSDME in B cells with the more severe cases of CRS, suggesting that pyroptotic cell death can, at least partially, be involved in the CRS observed in patients. Interestingly, the authors observed that, although non-modified $CD8^+$ Tc cells were able to eliminate tumour cells to the same extent as CAR T cells, the resulting supernatants did not induce release of IL-1β nor IL-6 by macrophages, suggesting that the increased affinity of CART cells to target cells induces a much stronger activation of the lymphocytes, inducing the release of larger amounts of PRF/GZMB which ultimately induce pyroptosis in tumour cells. This is a very attractive hypothesis that might help to understand and treat CRS during CART cell therapy. However, the authors did not convincingly demonstrate the contribution of PRF and GZMB to CRS, as they did not show the effect of the absence of either PRF or GZMB on CRS neither *in vitro* nor *in vivo*. Thus, further experiments will be required to confirm whether excessive cell death induced by GZMB in tumour cells during CART cell therapy might be detrimental and contribute to CRS.

On the other hand, Zhang *et al.* [134] described that GZMB released by NK cells, besides inducing cleavage of GSDME through activation of caspase 3, was also able to directly cleave and activate GSDME in a caspase-independent manner. Tumour cells expressing high levels of GSDME presented reduced tumour growth *in vivo,* due to enhanced tumour immune responses. Interestingly, the authors correlated their results with a decreased expression or the occurrence of loss of function mutations of GSDME in human cancer cells, which might have acquired these modifications as an evolutionary advantage to evade immunosurveillance. Thus, these complementary studies confirmed that GZMB might activate pyroptosis in some types of cancer cells, although its contribution to enhanced protective (immunogenic cell death and crosspriming of T-cell responses) or detrimental (CRS) immune responses is still not solved. Finally, Zhou *et al.* [135] demonstrated that GZMA released from NK and T-cell cleaves and activates GSDMB, triggering pyroptosis in a caspase-independent manner.

Altogether, these studies unveil a previously ignored activation of pyroptosis by CLs in tumour cells expressing GSDMs. *A priori*, activation of pyroptosis might be doubly beneficial: on the one hand, it is a faster mechanism than apoptosis, and therefore, cells expressing GSDMs will die in a quicker way than those which do not, and on the other hand, pyroptosis induces a strong pro-inflammatory signal by releasing DAMPS and cytokines, amplifying the immune activation and improving the antitumour response. However, it should also be taken into account the danger of uncontrolled pyroptotic cell death as it might contribute to inflammatory disorders like CRS, similarly to the effects of pyroptosis during sepsis [136]. In summary, more research will be required to clarify whether pyroptosis induced by CLs plays any physiological role during immunosurveillance or, on the contrary, it is a consequence of immune cell overactivation during immunotherapy.

Role of inflammatory cell death triggered by cytotoxic lymphocytes

As aforementioned, the immunogenic potential of different cell death modalities has been mainly studied in the context of radiotherapy, chemotherapy and infection. However, very few evidences are available regarding the immunogenicity of cell death induced by CLs and the mechanisms involved. In this line, and in parallel with Ignacio Melero's team, our group very recently showed that CLs (including antigen-specific $CD8^+$ Tc, transgenic TCR-expressing Tc cells and NK cells) were able to induce immunogenic cell death by crosspriming a $CD8^+$ T-cell-dependent response against dying cells that protected against subsequent tumour challenge [9,10]. This finding explained the phenomena of antigen spread observed during different immunotherapy treatments such as vaccines or CART cells. Intriguingly, we found that caspase 3 activity was necessary for the induction of immunogenic cell death by $CD8^+$ Tc cells in a model of EL4 tumour cells, although it was not required for the induction of cell death [10]. At that time, that finding seemed counterintuitive, as apoptosis was always considered as an immunologically silent form of cell death. However, and considering the recent

findings summarized in this review, it is tempting to speculate that caspase 3 activity was required for the cleavage of a GSDM (possibly GSDME) in EL4 cells, which would trigger the release of pro-inflammatory factors, increasing the immunogenicity of the cell death. Further experimental work will be required to reveal the role of caspase 3 in immunogenic cell death induced by CLs, and the relative contribution of apoptosis and pyroptosis in this process.

Importantly, the strong inflammatory response triggered by a minority pyroptosis within a tumour might be sufficient to mount a strong immune response against the rest of the tumour cells. In this regard, Wang *et al*. [137] have shown how induction of pyroptosis using nanoparticles releasing active GSDMA3 selectively into tumour cells was able to prime a strong antitumour response elicited by T cells. In their study, nanoparticle-delivered GSDMA3 was only able to induce pyroptosis in less than 15% of cells in a model of xenografted breast cancer using 4T1 cells. However, this initial pyroptosis was sufficient to trigger a robust antitumour immune response in combination with checkpoint blockade through release of IL-1β and to a lesser extent IL-18. Ultimately, the antitumour response resulted in the total elimination of the tumour, illustrating the power of pyroptosis at inducing immune activation, and the potential physiological relevance that a minority pyroptotic cell death triggered by cytotoxic lymphocytes might play physiologically during immunosurveillance.

Nevertheless, there are several caveats that require further investigation. An important issue is the reduced expression of GSDMs by tumour cells [65]. In this regard, Zhou *et al*. [135] showed how stimulation with IFNγ (a cytokine mainly secreted by activated NK cells and T cells) was able to induce GSDMB expression in around 30% of cell lines tested, enabling GZMA-mediated pyroptosis. Alternatively, this problem could also be circumvented by treatment with epigenetic drugs such as decitabine, a drug approved for treatment of leukaemia, which might induce expression of GSDMs [93]. On the other hand, care must be taken with regard to the strength of the pro-inflammatory signal triggered by pyroptosis. It seems clear that CLs mediated pyroptosis can provide a strong pro-inflammatory signal that can greatly boost the antitumour immune response. However, it is also true that too much pyroptosis can be detrimental, as exemplified by the GSDME-mediated CRS occurring during CART cell therapy [133]. Use of modified CART cells engineered to restrict their activation, or co-treatment with anti-inflammatory drugs within a therapeutic window able to control a potentially harmful inflammatory response could help circumvent this problem, although further investigation in this regard is necessary.

Conclusion

Under the light of the most recent advances in the cell death field, we must reconsider the importance of inflammatory cell death for the successful activation of the immune system and for triggering a strong antitumour response. Moreover, the discovery of NK cells and Tc cells inducing pyroptosis, necroptosis and ferroptosis in addition to apoptosis highlights the potential physiological relevance of these forms of regulated necrosis in cancer immunosurveillance and in immunotherapy. The activation of these highly inflammatory forms of cell death enables a quick and effective way to boost the immune response. However, caution must be taken regarding an excessive inflammatory response caused by overactivation of CLs, as exemplified by the severe inflammatory syndromes observed in some patients treated with CART cell therapy. We need to advance in our understanding of cytotoxic immune cells, and how we can harness and maximize in a controlled manner the cytotoxic potential of these cells whilst avoiding the deleterious inflammatory side effects of triggering uncontrolled cell death pathways.

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Conflict of interest

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Author contributions

DDM elaborated the manuscript and the figures, and curated the bibliography; AR-L, MA, IU, SH, LS, and EMG participated during the draft elaboration and review of the manuscript; JP provided the main view, revised the elaboration process and finalized the manuscript.

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