



# Escaping Death: How Cancer Cells and Infected Cells Resist Cell-Mediated Cytotoxicity

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
T Cell Biology,  
a section of the journal  
Frontiers in Immunology

**Received:** 31 January 2022

**Accepted:** 04 March 2022

**Published:** 23 March 2022

### Citation:

Tuomela K, Ambrose AR and  
Davis DM (2022) Escaping Death:  
How Cancer Cells and Infected Cells  
Resist Cell-Mediated Cytotoxicity.  
*Front. Immunol.* 13:867098.  
doi: 10.3389/fimmu.2022.867098

Cytotoxic lymphocytes are critical in our immune defence against cancer and infection. Cytotoxic T lymphocytes and Natural Killer cells can directly lyse malignant or infected cells in at least two ways: granule-mediated cytotoxicity, involving perforin and granzyme B, or death receptor-mediated cytotoxicity, involving the death receptor ligands, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL). In either case, a multi-step pathway is triggered to facilitate lysis, relying on active pro-death processes and signalling within the target cell. Because of this reliance on an active response from the target cell, each mechanism of cell-mediated killing can be manipulated by malignant and infected cells to evade cytolytic death. Here, we review the mechanisms of cell-mediated cytotoxicity and examine how cells may evade these cytolytic processes. This includes resistance to perforin through degradation or reduced pore formation, resistance to granzyme B through inhibition or autophagy, and resistance to death receptors through inhibition of downstream signalling or changes in protein expression. We also consider the importance of tumour necrosis factor (TNF)-induced cytotoxicity and resistance mechanisms against this pathway. Altogether, it is clear that target cells are not passive bystanders to cell-mediated cytotoxicity and resistance mechanisms can significantly constrain immune cell-mediated killing. Understanding these processes of immune evasion may lead to novel ideas for medical intervention.

**Keywords:** cell-mediated cytotoxicity, cytolytic T cells, natural killer cells, lymphocytes, cancer, resistance, viral infection, immune synapse

## INTRODUCTION

Cytotoxic lymphocytes, including cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, are able to directly lyse malignant or infected cells using multiple mechanisms. Granule-mediated cytotoxicity involves the release of lytic granules containing perforin and granzymes, while death receptor-mediated cytotoxicity utilises tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) or Fas ligand (FasL) that bind death receptors on the surface of the target cell (1–3). In addition to these classic cytotoxic pathways, there is increasing evidence that the tumour necrosis factor (TNF) pathway also significantly contributes to lymphocyte cytotoxicity (4). Activation of these pathways can lead to cell death in several forms, including necrosis, apoptosis, necroptosis, and pyroptosis (5).

Lymphocyte cytotoxicity is triggered upon contact with a cancerous or infected target cell if sufficient activating signals are received. For CTLs, this requires initial priming by antigen presenting cells followed by T cell receptor recognition of specific target cell antigens, whereas activating receptors on NK cells recognise a range of germline-encoded ligands without prior activation (6, 7). The area of cell-cell contact between a lymphocyte and a target cell is termed an immune synapse, on account of it being a highly organised interface involving cytoskeletal and membrane rearrangement (8–12). Integration of multiple activating and inhibitory pathways within the lymphocyte determines the outcome of this interaction with a target cell (13, 14). Malignant or infected cells may, therefore, evade immune recognition through the downregulation of activating signals or the upregulation of inhibitory signals. These immune evasion mechanisms – for example reducing antigen recognition, triggering immune checkpoints, secreting immunosuppressive cytokines, as well as excluding immune cells from the microenvironment – have been extensively reviewed elsewhere (15–18). And of course, many immunotherapies have been developed to target these types of immune escape mechanisms, such as checkpoint inhibitors, adoptive cell therapy, and cancer vaccines, all of which aim to enhance immune cell activation (6, 19).

However, evasion of lymphocyte cytotoxicity may also occur downstream of immune cell activation. Even if a cytolytic response is triggered by the lymphocyte, target cells are not passive bystanders to lymphocyte cytotoxicity. This is because mechanisms of cell death generally rely on active pro-death signalling within the target cell (5). Indeed, evasion of cell death and apoptosis is considered a critical hallmark of cancer (20). Therefore, resistance to lymphocyte attack may arise through resistance to the mediators of cytotoxicity, including perforin, granzymes, and death receptor ligands. Here we review the molecular details behind cell-mediated killing and then examine our current understanding of how target cells may resist these cytotoxic processes as immune evasion strategies.

## MECHANISMS OF LYMPHOCYTE CYTOTOXICITY

### Granule-Mediated Cytotoxicity

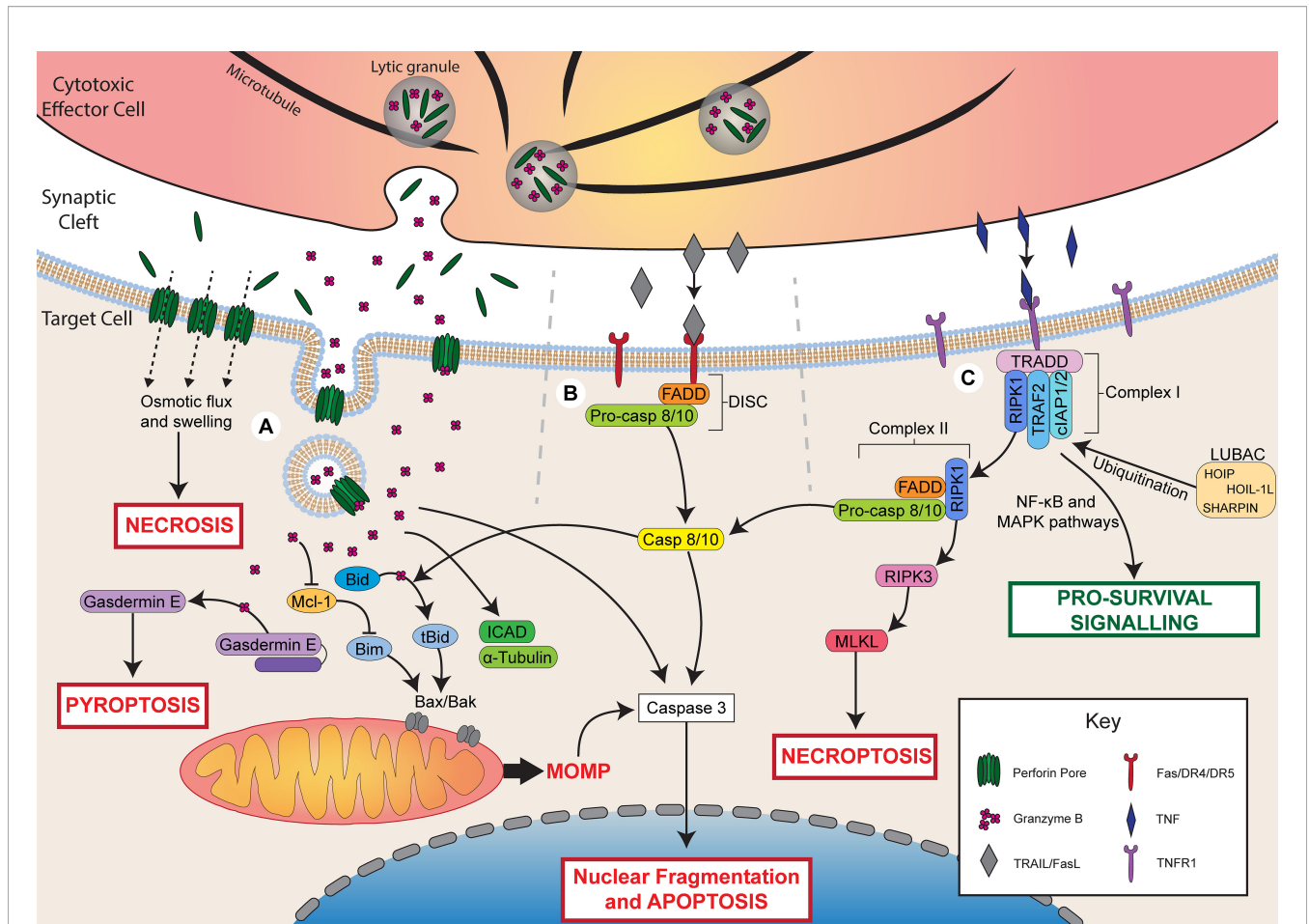
Following activation, effector cells polarise their microtubule organising centre (MTOC) and lytic granules towards the immune synapse, then release the contents of these granules across the synaptic cleft (21–23). Granule-mediated cytotoxicity is dependent upon the release of perforin and granzymes from granules contained within cytotoxic lymphocytes (**Figure 1**). Perforin is a pore-forming protein that forms ring-shaped lesions capable of mediating ion flux as well as the uptake of larger molecules, such as granzymes (24). Granzymes are a family of serine proteases that cleave a variety of target proteins within cells in order to induce apoptosis. Five granzymes have been identified in humans, A, B, H, K, and M,

but granzyme A and B have been characterised most extensively (25, 26).

Granule-mediated cytotoxicity can result in cell death through two mechanisms (**Figure 1**). The first is necrotic cell death induced by rapid osmotic flux through perforin pores and membrane rupture, which can be observed upon exposure to high concentrations of perforin (27, 28). The second is apoptotic cell death induced by perforin-mediated uptake of granzyme B into the target cell. Two primary models have been proposed to account for the entry of granzyme B into target cells: direct diffusion through perforin pores in the cell membrane, or perforin-induced endosomal uptake of granzymes. Perforin pores observed by electron microscopy have been measured to be physically large enough to permit the diffusion of granzymes into the target cell cytoplasm (29). Furthermore, intracellular granzyme B activity can be observed within minutes of adding exogenous granzyme B and perforin to target cells (30), which is faster than has been observed for endosomal uptake of granzyme B (31). Conversely, perforin has been shown to trigger a calcium-dependent membrane repair response that triggers the endocytosis of granzyme B, and granzyme B-positive endosomes can be observed within target cells following interaction with NK cells (31–33). It is possible that these two pathways work in parallel or in different cellular contexts to facilitate the uptake of granzyme B into target cells. Recent evidence demonstrated that perforin and granzyme can also be secreted from CTLs and NK cells in complexes, termed supramolecular attack particles (SMAPs), bound by the adhesive glycoprotein thrombospondin-1 (34, 35). Further understanding of SMAP biology may shed light on whether or not the two models of granzyme B delivery work synergistically or independently.

Once within a target cell, granzymes can trigger cell death through several pathways, and the specific pathways that are activated are dependent on the identity of the granzyme. Granzyme B is the most potent member of the granzyme family and can induce apoptosis within minutes of delivery (30). This occurs through either direct cleavage of caspases by granzyme B or *via* activation of the mitochondrial pathway of apoptosis. Direct cleavage of caspases, such as caspase 3, one of the executioner caspases, is the primary mechanism by which mouse granzyme B induces apoptosis (25). Caspase 3 cleaves several targets including inhibitor of caspase-activated DNase (ICAD) and gelsolin, which leads to DNA damage and cytoskeletal disruption, respectively (36). Both human and mouse granzyme B can also trigger the mitochondrial apoptotic pathway, which is characterised by mitochondrial outer membrane permeabilization (MOMP) (37–39).

MOMP is regulated by the Bcl-2 family, which is made up of three classes: pro-apoptotic BH3 proteins (e.g. Bid, Bim), pro-apoptotic effector proteins (e.g. Bax, Bak), and anti-apoptotic proteins (e.g. Bcl-2) (40). Granzyme B triggers MOMP by cleaving Bid into its active, truncated form (tBid) (37). After cleavage, tBid recruits the pore-forming effector proteins, Bax and Bak, to the mitochondrial membrane where they form pores that mediate MOMP and the release of additional apoptotic mediators, such as cytochrome c (40). This process can be



**FIGURE 1** | Mechanisms of lymphocyte cytotoxicity. Following activation, cytotoxic effector cells can kill through granule-mediated cytotoxicity, death receptor-mediated cytotoxicity, or TNF-mediated cytotoxicity. **(A)** During granule-mediated cytotoxicity, perforin and granzymes are released from lytic granules into the synaptic cleft. Perforin forms pores in the target cell membrane. At high concentrations of perforin, osmotic flux through pores leads to cell swelling and necrotic cell death. Perforin can facilitate the uptake of granzyme B through direct diffusion or endocytosis. Granzyme B directly cleaves caspase 3 to induce apoptosis or triggers the mitochondrial apoptotic pathway via Bid cleavage into tBid. tBid recruits Bax/Bak leading to mitochondrial outer membrane permeabilization (MOMP) and apoptosis. Granzyme B may also degrade Mcl-1 releasing Bim to activate MOMP. Granzyme B can also cleave ICAD contributing to DNA damage,  $\alpha$ -tubulin leading to cytoskeletal degradation, or gasdermin E, which forms pores in the cell membrane to induce pyroptosis. **(B)** Ligation of death receptors (Fas/DR4/DR5) by FasL or TRAIL triggers assembly of the death-inducing signalling complex (DISC) composed of FADD and pro-caspase 8/10. Caspase 8/10 induces apoptosis via direct caspase 3 cleavage or the mitochondrial apoptotic pathway via Bid cleavage. **(C)** Ligation of TNFR1 by TNF triggers the assembly of complex I (TRADD, RIPK1, TRAF2, cIAP1/2). LUBAC ubiquitinates complex I components leading to pro-survival signalling via NF- $\kappa$ B and MAPK pathways. In the absence of ubiquitination, RIPK1 dissociates and forms complex II with FADD and pro-caspase 8/10. Cleavage of pro-caspase 8/10 triggers apoptosis by the same pathways as FasL/TRAIL. In the presence of insufficient pro-caspase 8, RIPK1 can also recruit RIPK3, which activates MLKL to trigger necroptosis.

opposed by the action of anti-apoptotic Bcl-2 proteins, which bind and inhibit pro-apoptotic Bcl-2 members (40). Importantly, granzyme B can also induce degradation of these anti-apoptotic Bcl-2 proteins, such as Mcl-1, leading to the release of the proapoptotic BH3 protein, Bim, which activates Bax/Bak and triggers apoptosis (38, 41). A granzyme B-mediated mitochondrial apoptotic pathway, independent of Bax/Bak, has also been identified, and occurs through cleavage of mitochondrial proteins involved in the electron transport chain and production of reactive oxygen species (42–44).

Granzyme B can also cleave several additional targets that contribute to cell death. For example, granzyme B can directly

cleave the caspase 3 substrate, ICAD, leading to DNA damage (45) or cleave  $\alpha$ -tubulin, causing cytoskeletal disruption during apoptosis (46, 47). Recently, granzyme B was also found to cleave and activate the pore-forming protein gasdermin E, leading to an alternate form of cell death, pyroptosis, through the formation of gasdermin pores in the cell membrane (48, 49). Pyroptosis is a more inflammatory form of cell death compared to apoptosis and relies on the formation of pores in the cell membrane by members of the gasdermin family (5, 50). Cleavage of gasdermin E by granzyme B is a potent mechanism by which cytotoxic lymphocytes can kill cancer cells and control tumour growth (48).

Compared to granzyme B, granzyme A is a far less efficient inducer of cell death and triggers apoptosis at a slower rate (51). Granzyme A-induced apoptosis is caspase-independent and, although its targets are not fully defined, is mediated by cleavage of a variety of nuclear, mitochondrial, and cytosolic proteins (52, 53). Recently, granzyme A was also shown to trigger pyroptosis of cancer cells through cleavage of the pore-forming protein, gasdermin B (54, 55). In murine tumours, gasdermin B expression synergised with checkpoint blockade to promote tumour clearance by cytotoxic lymphocytes (54). Likewise, in the context of infection by *Shigella flexneri*, granzyme A secreted by NK cells was found to cleave gasdermin B within the infected cell (55). Cleaved gasdermin B demonstrated microbiocidal activity by forming pores within the bacterial membrane in order to protect the host cell. Less is known about the function and role of the other granzymes (K, H, and M) expressed by human lymphocytes.

### Death Receptor-Mediated Cytotoxicity

Cytotoxic lymphocytes may also kill target cells through the expression of ligands for death receptors. Two prototypical ligands have been identified that mediate apoptosis: FasL, which binds the Fas receptor, and TRAIL, which binds death receptors 4 and 5 (DR4/5) (56). Although FasL and TRAIL bind different receptors, both ligands trigger similar pro-apoptotic signalling (Figure 1). Both FasL and TRAIL are transmembrane proteins that belong to the TNF superfamily and can be expressed on cytotoxic immune cells upon cytokine stimulation or interaction with a target cell (1, 57). Upon binding of FasL or TRAIL to their respective receptors, assembly of the death-inducing signalling complex (DISC) is triggered. The DISC consists of the death receptor, Fas-associated death domain protein (FADD), and pro-caspase 8 or 10 (56). The DISC mediates cleavage of pro-caspase 8/10 to release the active caspase, which can then activate the executioner caspases 3, 6, and 7. Caspase 8 can further amplify apoptotic signalling by cleaving Bid to activate the mitochondrial pathway of apoptosis, similar to granzyme B (56).

### TNF-Mediated Cytotoxicity

TNF is a cytokine capable of inducing both pro-survival and pro-death signalling depending on the precise cellular context. Although the receptors for TNF, TNF-R1 and TNF-R2, belong to the same family as the receptors for FasL and TRAIL, the downstream signalling pathways are distinct (4). Of the two receptors for TNF, only TNF-R1 is able to trigger cell death through its cytoplasmic death domain, which recruits a key adaptor protein, TNF receptor-associated death domain (TRADD) (4). Conversely, both TNF-R1 and TNF-R2 contain a TNFR-associated factor (TRAF) binding site that recruits TRAF1/2, which is involved in triggering pro-survival signalling via the NF- $\kappa$ B and MAPK pathways.

The pro-survival and pro-death signalling pathways controlled by TNF-R1 ligation are mediated by the assembly of two signalling complexes, complex I and II, respectively (4, 58). Complex I, which mediates pro-survival signalling, is composed of several proteins, including receptor interacting serine/

threonine protein kinase 1 (RIPK1), TRAF2/5, cellular inhibitor of apoptosis 1/2 (cIAP1/2), and linear Ub chain assembly complex (LUBAC). LUBAC-mediated ubiquitination of complex I components leads to the recruitment of additional kinase complexes involved in NF- $\kappa$ B and MAPK survival signalling (4). Survival signalling through complex I is generally the default pathway triggered by TNF-R1 ligation. However, in certain cell states, TNF-R1 signalling can switch instead to pro-death signalling mediated by complex II, which is composed of RIPK1, FADD, and pro-caspase 8 (4, 58, 59). Assembly of complex II occurs when RIPK1 is not ubiquitinated, such as in the absence of the complex I-associated proteins cIAP1/2 and LUBAC (59, 60). Non-ubiquitinated RIPK1 dissociates from TRADD and recruits FADD and pro-caspase 8 leading to similar pro-death signalling as TRAIL/FasL (4). When caspase 8 activation is not sufficient, complex II can also lead to necroptotic cell death. This occurs through autophosphorylation of RIPK1, leading to the recruitment and autophosphorylation of RIPK3 followed by activation of mixed lineage kinase domain-like (MLKL), which induces necroptosis (4). Altogether, much remains to be understood about the signalling that regulates the varying effects of TNF and how this can change in different cell states.

## MECHANISMS OF RESISTANCE TO CYTOTOXICITY

Overall, there is a reliance on active processes and signalling pathways within the target cell to execute CTL and NK cell killing. This implies that each distinct mechanism of cell-mediated killing can be open to an evasion strategy by the target cell. Indeed, malignant and infected cells develop a variety of mechanisms to evade cytolytic death.

The existence of these resistance mechanisms is readily observed when tracking interactions between cytotoxic lymphocytes and target cells *in vitro*. Even when CTLs are activated during an interaction with a target cell – as indicated by a rapid increase in calcium concentration within the effector cell – the target cell does not always die (61, 62). In some cases, CTLs may produce a sublethal hit, which is characterised by a transient calcium flux, indicative of perforin pore formation, but no cell death (62). Alternatively, in some effector-target interactions, no calcium flux could be observed in the target cell despite apparent activation of the effector cell, indicating that perforin did not form pores in the target cell membrane. A similar study demonstrated that cancer cells often recover even after a cytotoxic hit that triggers a large calcium flux, structural perturbations, and DNA damage (61). The target cancer cells were observed to rapidly restore calcium homeostasis, recover nuclear integrity after structural damage, and even repair DNA to stop apoptosis at its later stages (61). Clearly, not every interaction between a cytotoxic lymphocyte and its target results in death. While some of this survival could be attributed to stochastic variability, specific methods by which target cells can evade cytotoxicity can have a profound impact on

the ability of cytotoxic lymphocytes to eliminate cancer cells and infected cells *in vitro*.

## Resisting Perforin Pore-Formation

The formation of perforin pores in target cell membranes is the first step of granule-mediated cytotoxicity and is required for the induction of both necrosis caused by osmotic flux and apoptosis caused by granzyme B uptake. Therefore, resistance to this initial step of granule-mediated cytotoxicity has the potential to significantly reduce lymphocyte cytotoxicity. Perforin resistance was initially identified as a characteristic of cytotoxic lymphocytes, including CTLs and NK cells, which are less easily killed by purified perforin compared to various non-cytotoxic cell lines (63–65). This resistance is thought to be integral to the survival of lymphocytes when releasing their cytotoxic cargo. Resistance to perforin has also been observed in malignant cells (66–68). In studies of patient-derived leukaemia and lymphoma samples, considerable variability was observed in the ability of perforin to bind and lyse cancer cells from different patients (66, 67). Importantly, the susceptibility of cancer cells to perforin-induced lysis closely correlated with the amount of perforin bound (66), indicating that cancer cells may evade perforin by reducing binding. More recently, our own research has found that *in vitro* irradiation of cancer cells transiently reduces susceptibility to lysis by NK cells and CAR T cells by inducing resistance to perforin, possibly by preventing pore formation (68). Our current understanding of the mechanisms that mediate perforin resistance in malignant or infected cells is predominantly derived from protective mechanisms employed by cytotoxic lymphocytes. These mechanisms of perforin resistance include altered lipid order, phosphatidylserine exposure, modulation of cell stiffness, and cathepsin B-mediated degradation (**Figure 2**), each of which we will now explore in detail.

### Lipid Order

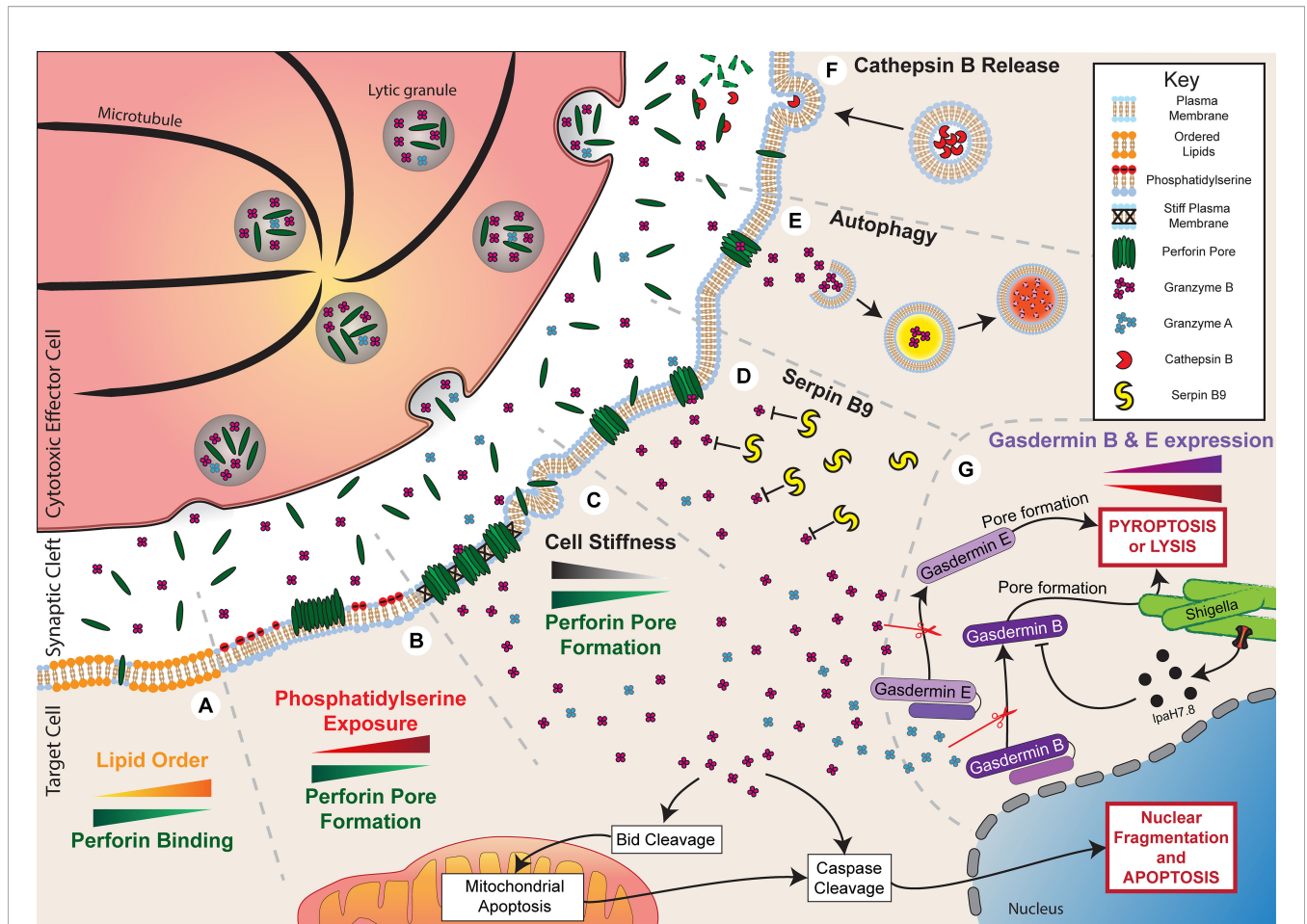
The lipid order of a membrane is a characteristic determined by several properties: lipid packing, the rotational freedom of lipids, and the thickness of the bilayer (69). Therefore, changes to membrane composition can alter lipid order, such as increasing lipid order upon incorporation of cholesterol (70). From the time of its discovery, perforin has been known to preferentially bind to synthetic liposomes or planar lipid bilayers composed of low-order, fluid-phase lipids, such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), compared to high-order, gel-phase lipids, such as dipalmitoylphosphatidylcholine (DPPC) (71–74). This specificity for lipid order appears to play a critical role in protecting cytotoxic lymphocytes against self-harm by perforin since CTLs have particularly tightly packed and ordered membranes (72, 73). Lipid order has also been observed to particularly increase at the site of the immune synapse compared to more distal areas of the lymphocyte membrane (75–77). Replacing cholesterol in CTL membranes with a disorder-prone cholesterol variant increases perforin binding and sensitises cells to pore-induced lysis, consistent with tight lipid packing being protective against perforin pore formation (73).

Alterations in lipid packing may directly affect the susceptibility of cancer cells to perforin-mediated attack. Lymphocyte-resistant breast cancer cells, for example, have been found to increase lipid order at the site of the immune synapse and, similar to observations with CTLs, replacing cholesterol with a disorder-prone variant sensitises cancer cells to perforin-induced lysis (73, 75). More broadly, lipid composition is often highly altered during malignancy and infection. For example, multidrug resistant cancer cells frequently exhibit increased membrane lipid order due to increased cholesterol levels (78, 79). Therefore, it is possible that lipid order-mediated resistance to perforin is a common feature of cancer. However, most observations have been made *in vitro* and further work is necessary to understand whether alterations in the lipid order of cancer cell membranes can have a significant impact on tumour control by lymphocytes *in vivo*.

### Phosphatidylserine Externalisation

Apart from altering lipid order, membrane composition can affect perforin activity through other mechanisms. Phosphatidylserine is a negatively charged phospholipid that generally localises to the intracellular leaflet of the cell membrane but can be externalised to the outer leaflet in certain cell states (80). In particular, externalisation of phosphatidylserine on the outer leaflet is often used as a marker of cell death, but it also has a variety of non-apoptotic roles for intercellular signalling (81). Importantly, phosphatidylserine can be externalised on lymphocyte membranes at the immune synapse, where it is suggested to act as a protective mechanism against perforin pore formation (73, 74, 82). Atomic force microscopy has shown that perforin is able to bind to phosphatidylserine-containing planar lipid bilayers, but it forms protein aggregates rather than membrane-spanning pores (73, 74). In addition, perforin shows little or no lytic activity against synthetic liposomes composed of high levels of phosphatidylserine (68). Other negatively charged membrane lipids, such as 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) or cholesterol sulfate, had a similar effect in preventing perforin pore formation, suggesting that the negative charge of phosphatidylserine is critical for its inhibitory effect against perforin (74).

In addition to protecting cytotoxic lymphocytes, phosphatidylserine may also be utilised by infected or malignant cells to evade attack mediated by perforin. Phosphatidylserine exposure is a common feature of cancer cells and can be further enhanced in certain circumstances, such as following anti-cancer treatment (68, 83). Externalisation of phosphatidylserine following the treatment of cancer cells with radiotherapy or cell cycle inhibitors was found to closely correlate with resistance to perforin and lymphocyte cytotoxicity, despite normal recognition and activation by NK cells and CAR T cells (68). Treatment of cancer cells with radiotherapy or cell cycle inhibitors did not affect perforin binding or membrane repair responses, suggesting that the mechanism of resistance was impaired pore formation, similar to the observed effects of phosphatidylserine in synthetic lipid membranes (68, 73, 74). Increased surface phosphatidylserine on malaria-infected erythrocytes also correlated with reduced



**FIGURE 2 |** Resistance to granule-mediated cytotoxicity. Activation of a cytotoxic effector cell at an immune synapse with a target cell leads to the polarisation and secretion of lytic granules containing perforin and granzyme B. Under normal circumstances, in the absence of any resistance mechanisms, the secreted perforin will form pores in the target cell membrane and allow entry of granzyme B. This process will initiate cell death through both direct cell lysis and the activation of apoptotic pathways. Target cells can employ multiple mechanisms to evade cytotoxicity. **(A)** Increased plasma membrane lipid order to reduce perforin binding. **(B)** Externalisation of phosphatidylserine to induce perforin aggregation rather than pore formation. **(C)** Reduced cell stiffness to prevent efficient perforin pore formation. **(D)** Expression of Serpin B9 to directly inhibit Granzyme B activity. **(E)** Autophagy of Granzyme B to prevent activation of apoptotic pathways. **(F)** Secretion of Cathepsin B to degrade perforin. **(G)** Reduced gasdermin B and E expression or IpaH7.8-mediated ubiquitination and degradation of gasdermin B can reduce pyroptosis or lysis of shigella, respectively.

susceptibility to perforin and reduced lysis by  $\gamma\delta$  T cells (84). The extent to which phosphatidylserine externalisation affects the elimination of target cells *in vivo* is unclear, and hard to establish. Whether this process could be targeted therapeutically is an open question.

**Cell Stiffness**

Physical properties of cells may also influence susceptibility to perforin, including cell tension or stiffness. Cell stiffness is commonly altered during malignancy, with cancer cells being relatively soft and deformable compared to healthy cells (85). Accordingly, soft CD133+ tumour-repopulating cells were found to be resistant to perforin and take up less granzyme B following interaction with T cells (86). These cells also evaded T cell cytotoxicity *in vivo* but killing could be enhanced if cells were treated with jasplakinolide, which promotes actin polymerisation

and increases cancer cell stiffness. Cell stiffness can also be artificially altered by culturing cells on stiff or soft hydrogels, which directly changes the stiffness of cells to mirror the underlying substrate. Reducing stiffness in this way by culturing on a soft substrate has been shown to reduce susceptibility to perforin and lymphocyte cytotoxicity (87). It is not entirely clear why cell stiffness affects perforin lytic ability in this way, but insertion and pore formation by hydrophobic molecules, such as perforin, is known to be more energetically favourable on stiff membranes (87, 88).

Interestingly, cytotoxic lymphocytes have been found to utilise a mechanism which may counteract the reduced activity of perforin on soft cells. By using F-actin-rich protrusions, CTLs can exert lateral force on the target cell to increase membrane tension and enhance perforin pore formation (87, 89). Lytic granule secretion from CTLs was observed at the base of these

protrusions so that it was spatially localised to the areas of force exertion on the target cell membrane (89). It is interesting to note that the stiffness of a target cell can also modulate NK cell and CTL activation itself, with activation significantly reduced against target cells exhibiting a soft phenotype or grown on a soft substrate (90, 91). Thus, alterations in cell stiffness at the whole-cell level, such as during malignancy, or at the nanoscale, at the immune synapse, may potentially manipulate the sensitivity of cancerous or infected cells to perforin.

### Cathepsin B

The pore-forming activity of perforin may also be reduced through direct degradation. Cysteine cathepsins are lysosomal peptidases with multiple roles in regulating immune responses (92). It was initially shown that cathepsin B, which becomes expressed on the surface of lymphocytes following degranulation, can degrade perforin in order to protect against self-harm (93). Inhibition of this surface-bound cathepsin B using the inhibitor CA074 led to enhanced CTL death after degranulation. Seemingly in contrast to this, it was later shown that CTLs from cathepsin B-null mice were not more susceptible to death following interaction with a target cell compared to cells from wild type mice (94). To reconcile these observations, it is possible that there is redundancy in the system, and perhaps compensatory mechanisms are augmented in cathepsin B-null mice, such as increased lipid order or phosphatidylserine exposure, as discussed previously.

More recent evidence has also linked cathepsin B with cancer cell resistance against lymphocyte cytotoxicity. Khazen et al. (95) showed that melanoma cells that were resistant to CTL cytotoxicity bound less perforin and took up less granzyme B despite inducing similar levels of CTL degranulation. This resistance was mediated by increased exocytosis of lysosomes or late endosomes at the immune synapse, which facilitated the secretion of cathepsin B leading to perforin degradation. Interestingly, cathepsin B is known to be overexpressed in multiple cancers, where it is associated with poor survival and metastasis (96).

Altogether, resistance to perforin through physical or degradative inhibition is an emerging aspect of immune resistance. Many of the mechanisms employed by cytotoxic lymphocytes to protect against self-harm appear to also be exploited by malignant or infected cells to inhibit perforin activity and enhance survival. However, the contribution of perforin resistance to immune escape has not yet been extensively explored *in vivo*. The ability to modify cancer cells pharmacologically to increase perforin susceptibility may be a way to increase the efficacy of lymphocyte cytotoxicity.

### Resisting Granzyme-Mediated Apoptosis

In addition to perforin, granzymes are a critical component of granule-mediated cytotoxicity. Following perforin pore formation, granzymes enter the target cell and cleave a variety of targets to potentially induce cell death. As a result, reduced granzyme activity within target cells may critically constrain killing by lymphocytes. Granzyme activity may be reduced in two ways: through reduced granzyme uptake as a result of perforin

inhibition, as described previously, or through direct inhibition of granzyme function. The effect of reduced granzyme uptake on cytotoxicity was clearly demonstrated by the finding that cancer cells, which were resistant to NK cell cytotoxicity, can undergo extensive cytoskeletal remodelling, which reduces granzyme uptake (97, 98). Although the underlying link between cytoskeletal remodelling and reduced granzyme uptake was not identified in these studies, cytoskeletal inhibitors were found to restore granzyme levels and cytotoxicity following interaction with NK cells. Granzyme-induced cell death may also be reduced by direct inhibition of granzyme activity. Of all the proteins in the granzyme family, the mechanisms that reduce granzyme B-mediated cell death have been characterised the most. Inhibitory mechanisms that act directly on granzyme B pathways to prevent cell death include inhibition by serpin B9, degradation through autophagy, and disruption of gasdermin-mediated pyroptosis (Figure 2).

### Serpin B9

Serpin B9 (also known as serine proteinase inhibitor B9 or proteinase inhibitor 9) is the only endogenous granzyme B inhibitor that has been identified. It was initially discovered in cytotoxic lymphocytes where it protects against apoptosis by binding to and inhibiting granzyme B (99, 100). When unbound to substrate, serpin B9 exists in a semi-stable form, but it is cleaved upon binding to granzyme B causing a conformational change into its most stable form and leaving a non-functional covalently-bound serpin B9-granzyme B complex (101). Apart from in immune cells and at certain immune-privileged sites, such as reproductive organs and the eye, normal human tissue does not express serpin B9 (102). However, serpin B9 expression has been observed in multiple primary cancers, including lymphoma, melanoma, colon carcinoma, breast cancer, and lung cancer, in which it generally correlates with poor prognosis (102–107).

Overexpression of serpin B9 in various cancer cell lines results in resistance to killing by cytotoxic lymphocytes and, critically, is associated with resistance to immune checkpoint blockade in murine melanoma as well as against radiotherapy-induced type I interferon signalling (104, 108–112). Interestingly, the resistance of serpin B9-expressing cancer cells to cytotoxic lymphocytes is less evident at high ratios of lymphocytes to cancer cells (111). Furthermore, it has been shown through live imaging that multiple NK cell attacks successfully kill serpin B9-expressing target cells, while single hits are sufficient to kill targets which don't express serpin B9 (113). This is consistent with serpin B9-mediated inhibition of cytotoxicity being overcome through increased granzyme B delivery *via* multiple lytic hits.

Although serpin B9 has primarily been described as an inhibitor of lymphocyte-derived granzyme B, it has also been shown to have a broader role in mediating tumour immune escape. For example, overexpression of serpin B9 can inhibit TRAIL-, FasL-, and TNF-mediated apoptosis through directly inhibiting caspase 8 and 10 (108, 114). Furthermore, serpin B9 can promote tumour survival through inhibition of cancer cell-intrinsic granzyme B, which can become expressed in various malignancies (107). Therefore, pharmacological inhibition of

serpin B9 may aid the destruction of target cancer cells through multiple pathways. Recently, inhibition of serpin B9 has been shown to slow the development of melanoma and increase the lifespan of mice with breast, kidney and lung tumours (107).

### Autophagy

Cancer cells may also evade cytotoxicity through autophagic pathways. Autophagy is a physiological process by which damaged or surplus proteins and organelles are degraded and recycled (115). It has a particularly important role in preventing cell death during cellular stress, such as nutrient starvation or hypoxia. Increased autophagy is also a common feature of tumorigenesis to protect against the harsh environment often present within tumours. This also enables cancer cells to maintain their highly proliferative and metabolically active states even when the microenvironment is not conducive to it (115).

Autophagy may also contribute to tumour growth by promoting immune evasion. Hypoxia-induced autophagy, for example, has been shown to correlate with resistance to CTL and NK cell cytotoxicity (116, 117). Similarly, induction of autophagy as a result of genetic inactivation of the von Hippel-Lindau (VHL) gene reduces killing by NK cells (118). Genome-wide CRISPR screens searching for genes that mediate resistance to CTL cytotoxicity have also identified a range of autophagy-related genes associated with cytotoxicity resistance (119, 120). Degradation of granzyme B may be one mechanism by which autophagy inhibits cytotoxicity. Breast cancer cells with autophagy processes stimulated by hypoxia were found to be resistant to NK cell-mediated lysis, with granzyme B localising within their autophagosomes (117). When hypoxia-related genes were inhibited, granzyme B activity within target cells was increased (117, 118). However, a later study demonstrated that a major effect of autophagy in cancer cells is to inhibit TNF $\alpha$  and TRAIL-induced apoptosis by reducing FADD-dependent caspase-8 activation (120). Thus, it is possible that autophagy can act in multiple ways to inhibit lymphocyte cytotoxicity.

### Gasdermins

An emerging aspect of resistance to lymphocyte cytotoxicity is evasion of gasdermin-induced pyroptosis. Both granzyme A and B-mediated cleavage of gasdermin B and E, respectively, have been shown to contribute to tumour control by cytotoxic lymphocytes (48, 54). Furthermore, granzyme A-mediated cleavage of gasdermin B also contributes to defence against bacterial infection by NK cells (55). Several mechanisms have been identified which may cause resistance to these gasdermin-mediated cytotoxic pathways. Firstly, reduced expression of both gasdermin B and E have been identified in many cancers (48, 54). In the case of gasdermin E, reduced expression can occur through epigenetic silencing *via* hypermethylation of the promoter region (121, 122). Low expression of both gasdermin B and E is associated with poor survival in various cancers, including breast cancer, bladder cancer, and melanoma (48, 54). Reducing granzyme-induced pyroptosis through silencing of gasdermin E expression in cancer cells has been shown to contribute significantly to the escape of murine tumours from

cytotoxic lymphocytes and accelerate tumour growth (48). Similarly, resistance to gasdermin-mediated pyroptosis can occur through the expression of mutated gasdermin. One study found that 20 out of 22 gasdermin E mutations identified within cancer samples were associated with reduced pyroptosis in response to granzyme B (48).

In the context of bacterial infection, pyroptosis has been shown to be inhibited through degradation of gasdermin B (55). Degradation was mediated by a bacterial ubiquitin ligase IpaH7.8 secreted by the gram-negative bacterium, *Shigella flexneri*. IpaH7.8 was shown to ubiquitinate N-terminal gasdermin B after its cleavage by granzyme A, leading to its degradation. Expression of IpaH7.8 significantly constrained the bactericidal activity of NK cells (55).

In summary, target cells may evade granzyme B-mediated apoptosis through inhibition by serpin B9 or degradation by autophagy. In addition, resistance to gasdermin-mediated pyroptosis is emerging as another mechanism by which cells may evade granzyme-mediated cytotoxicity in the context of malignancy and infection. Importantly, all of these mechanisms can have effects beyond granzymes and can affect other pathways of lymphocyte cytotoxicity as well as cell survival in other contexts. Therefore, targeting these pathways may directly impact cancer and could enhance other modes of treatment, such as immune therapies.

### Inhibiting Death Receptor-Mediated Killing

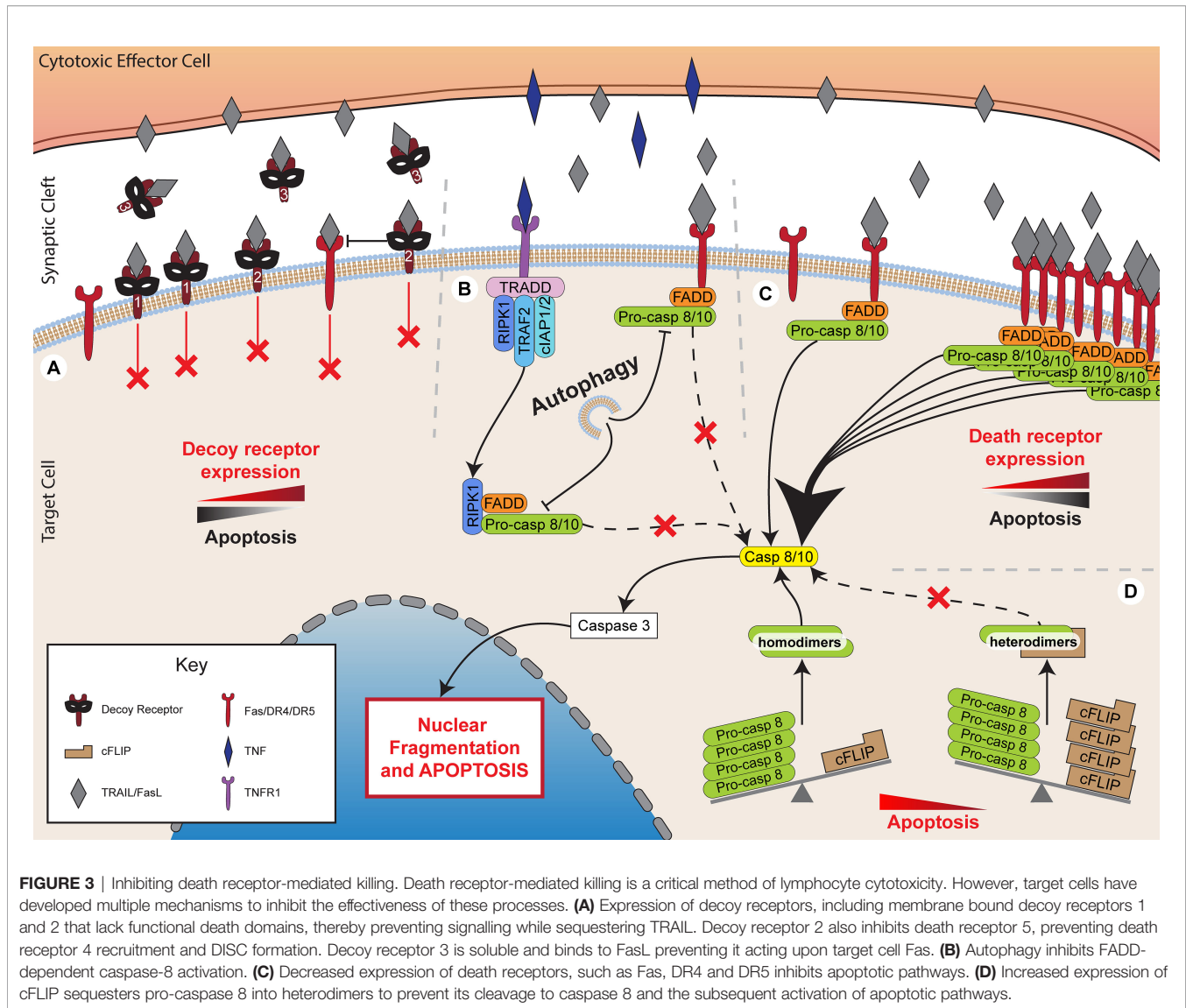
Death receptor-mediated cytotoxicity is another critical mechanism by which cytotoxic lymphocytes may eliminate target cells. Several mechanisms have been described by which cells can evade death receptor-mediated cytotoxicity. Signalling can be directly inhibited by the activity of FADD-like IL-1 $\beta$  converting enzyme (FLICE)-inhibitory proteins (FLIPs), expression of decoy receptors, or downregulation of death receptors (**Figure 3**).

#### FLICE Inhibitory Proteins (FLIPs)

One of the best characterised families of death receptor inhibitors are the FLIPs. This family of proteins includes both viral (v-FLIP) and cellular (c-FLIP) proteins, which share high sequence homology (123). Several FLIP splice variants are expressed in humans, but the primary forms include the short variant, c-FLIP<sub>S</sub>, and the long variant, c-FLIP<sub>L</sub> (123). The long variant contains an additional c-terminal domain that resembles the catalytic domains of caspase 8 and 10 but without functional caspase activity (123, 124).

Both cellular and viral FLIPs inhibit caspase 8 activity by forming heterodimers with pro-caspase 8 (123, 125). This sequesters pro-caspase 8, preventing it from forming the necessary homodimers required for processing into active caspase 8. Inhibition of pro-caspase 8 processing by FLIPs prevents apoptosis induced by TRAIL and FasL, but not by granzyme B (124, 126, 127). Immune cells have been observed to exert a selective pressure on cancer cells during *in vivo* tumorigenesis, allowing cells that highly express FLIP to escape (128). Indeed, high tumour expression of c-FLIP, particularly of the long variant, has been found to correlate with poor prognosis





in a range of cancers, including acute myeloid leukaemia, colorectal cancer, and non-small cell lung cancer (129–131).

In addition to cellular FLIPs, viral FLIPs also appear to have a role in the promotion of tumorigenesis in humans. v-FLIPs that are expressed during viral infection act to protect host cells from death receptor-induced apoptosis resulting in immune escape from T cells (132, 133). This viral immune escape mechanism can contribute to the process by which certain viruses are particularly oncogenic. For example, Kaposi's sarcoma-associated herpesvirus (KSHV)-FLIP is associated with Kaposi's sarcoma and certain lymphomas (132).

To complicate the picture, although c-FLIP<sub>L</sub> has primarily been described as an inhibitor of apoptosis, it can also have pro-apoptotic effects. Heterodimers formed of c-FLIP<sub>L</sub> and pro-caspase 8 have been found to retain their catalytic activity and can process other pro-caspase 8 homodimers (125, 134–137). There is evidence that whether or not c-FLIP<sub>L</sub> promotes

apoptosis is highly dependent on the level of expression of both the FLIP protein and pro-caspase 8 (125, 136, 137). In the presence of very high levels of c-FLIP<sub>L</sub>, and therefore high levels of heterodimers, inhibition of apoptosis occurs since the amount of pro-caspase 8 homodimers that are available to be processed is decreased. Conversely, at lower concentrations, c-FLIP<sub>L</sub> preferentially acts as a promoter of apoptosis.

Overall, FLIPs may play a significant role in the aetiology of some cancers. As a result, FLIP inhibitors or drugs that reduce FLIP expression are currently under development for the treatment of cancer, but balancing the pro- and anti-apoptotic effects may be challenging (138, 139). Other anti-cancer treatments, such as doxorubicin, synthetic triterpenoids, and peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) ligands, can also decrease FLIP expression as a side effect, therefore increasing sensitivity to death receptor-mediated cytotoxicity (140–142).

## Decoy Receptors

The majority of receptors that bind TNF superfamily proteins are capable of transducing signals. However, several receptors have been identified that, despite binding the same ligands, lack cytoplasmic death domains for signalling and cannot recruit critical adaptors, such as FADD (143). These are, in effect, decoy receptors which compete with functional death receptors for ligand binding. Physiologically, decoy receptors have been implicated in modulating inflammatory responses but have also been hijacked as a survival mechanism in cancer (144). One such protein is decoy receptor 3 (DcR3), a soluble receptor which binds FasL and is overexpressed in a large proportion of primary lung, colon, oesophageal, stomach, and rectal tumours (145, 146). Decoy receptors 1 and 2 (DcR1 and DcR2) are membrane-bound receptors which bind TRAIL and also lack a functional death domain (147, 148). In addition to competing with functional TRAIL receptors, DcR2 is also able to interact with TRAIL-receptor variant, DR5, preventing the recruitment of the DR4 variant to the DISC and inhibiting caspase activation (149, 150).

Expression of DcR1 or DcR2 has been found to correlate with tumour progression and poor prognosis in breast cancer, prostate cancer, and leukaemia (151–153). However, it is unclear whether targeting decoy receptors in tumours could have off-target effects, since they can also be expressed in several normal tissues, including the spleen, lung, gastrointestinal tract, endometrium, and activated T cells (145, 154, 155). Furthermore, although there is evidence from over-expression systems that decoy receptors may constrain death receptor-mediated killing, the extent to which they are harnessed by cancer cells to evade lymphocyte cytotoxicity *in vivo* is not known.

## Death Receptor Expression and Mutation

Death receptor signalling can also be lost in cancer cells through reduced surface expression or through inactivating mutations in death receptors. Reduced expression of the death receptors Fas or DR4/5 is a common feature of cancers (156–158). Loss of these receptors is associated with poor prognosis, particularly upon loss of more than one receptor or when receptor downregulation occurs in tumours with low levels of infiltrating CTL (156, 158). Interestingly, there is a weaker correlation between Fas expression and survival in colorectal tumours with high numbers of infiltrating CTL (158). This may suggest that granule-dependent cytotoxicity rather than death receptor-mediated killing is the predominant pathway of cancer cell elimination when large numbers of CTL are present (158). Loss of death receptor expression can occur through several routes, including promoter methylation (159–161), histone modifications (157, 162), promoter region mutations (163), or reduced trafficking to the cell membrane (164). Notably, oncogenic *Ras* mutations can strongly downregulate Fas expression through the control of several genes associated with the promoter region of *Fas*, as well as through hypermethylation (159, 160). Conversely, death receptors are often up-regulated as a side-effect of cancer treatment, and this may contribute to the overall efficacy of the treatment. For example, receptors for FasL

and TRAIL can be significantly up-regulated following radiotherapy and chemotherapy, leading to enhanced killing by cytotoxic lymphocytes (165–171).

A less frequently occurring feature of cancers that may contribute to immune escape is mutations of the death receptors themselves. Mutations affecting function, which generally localise in the cytoplasmic domains, are infrequently observed in cancers, such as gastric cancer, non-small cell lung cancer, metastatic breast cancer, non-Hodgkin's lymphoma, and head and neck cancer (172–175). Induced expression of these mutated receptors *in vitro* can reduce pro-apoptotic signalling.

Overall, death receptor signalling can be inhibited at multiple stages including reduced expression or mutation of death receptors, competition for ligand binding by decoy receptors, or inhibition of downstream signalling by FLIPs. However, there are several therapeutic strategies that may enhance death receptor signalling including pharmacological inhibition and downregulation of FLIPs or increasing death receptor expression, all of which have the potential to restore the efficacy of immune cytotoxicity.

## Inhibiting TNF-Mediated Cytotoxicity

TNF is known to have both pro-survival and pro-death effects on cancer cells depending on its precise cellular context. Recently, genome-wide CRISPR screens have identified TNF signalling as a major target of resistance to lymphocyte cytotoxicity (120, 176–179). These studies identified several genes encoding proteins related to TNF signalling that either sensitise cells to lymphocyte cytotoxicity – including TNF-R1, caspase 8, TRADD, and RIPK1 – or promote evasion of cytotoxicity – including TRAF2, cIAP1, and FADD-like apoptosis regulator (CFLAR), as well as multiple genes involved in the NF- $\kappa$ B pathway (120, 176–179). In particular, knockout of TRAF2 was shown to redirect TNF signalling from pro-survival signalling, *via* complex I proteins and the NF- $\kappa$ B pathway, to pro-death signalling, *via* complex II proteins (177). Likewise, knockout or pharmacological inhibition of HOIL-1-interacting protein (HOIP), the catalytic subunit of LUBAC involved in ubiquitination, also enhanced sensitivity to TNF by reducing the ubiquitination of pro-survival complex I proteins, which is required for TNF-mediated survival signalling (180, 181). Conversely, antibody blockade of TNF significantly reduced killing by both wild-type and perforin-deficient T cells demonstrating that TNF signalling is a major pathway of lymphocyte cytotoxicity (120, 176).

Resistance to TNF-mediated cell death has also been suggested as one way in which autophagy can induce resistance to lymphocyte cytotoxicity. Knockout of key autophagy-related genes sensitizes cancer cells to TNF-induced death and TNF-mediated T cell cytotoxicity (119, 120). Autophagy can target TNF-induced cell death by modulating FADD/caspase-8 activity (120). Several other studies have also noted the ability of autophagy to interfere with active caspase-8 leading to reduced susceptibility of cells to TRAIL and TNF during hepatic injury or in colon carcinoma (182, 183).

It remains to be seen whether TNF signalling can be harnessed to successfully treat cancer patients in the clinic because its effects are highly context-dependent. This was

demonstrated by Young *et al.*, who found that knockout of TNF-R1 could either protect tumours against immune checkpoint blockade or sensitise tumours to it depending on whether autophagy was impaired or intact, respectively (120). TNF has also been shown to have cancer-promoting effects in some cancer models, leading to several clinical trials of TNF antagonists demonstrating efficacy in some patients (184–186). One possible approach to targeting TNF signalling is through the use of SMAC (second mitochondria-derived activator of caspases) mimetics. These are drugs that mimic the activity of SMAC, a protein that is an endogenous inhibitor of cIAP function (187). Through the inhibition of cIAP, SMAC mimetics have been found to sensitise cancer cells to TNF-induced cell death both *in vitro* and *in vivo* (120, 188–190). Alternatively, directly inhibiting certain complex I components, such as HOIP, may redirect signalling towards apoptosis (180). Therefore, there is potential for sensitising cancer cells to TNF-induced cell death, but greater understanding of how its functions vary is needed.

## Inhibiting Apoptotic Pathways

In addition to the inhibitory mechanisms against specific components of lymphocyte cytotoxicity, cancer cells may exhibit more general resistance to apoptosis through alterations in apoptotic pathways. Prevention of apoptosis may occur through either down-regulation of pro-apoptotic mediators, such as caspases or pro-apoptotic Bcl-2 family members, or up-regulation of apoptosis inhibitors, such as Inhibitor of Apoptosis Proteins (IAPs) or anti-apoptotic Bcl-2 family members. These can affect both caspase-dependent and mitochondrial pathways of apoptosis, which are involved in both granule-mediated and death receptor-mediated cell death.

### Caspase Inhibition – Mutations and Inhibitor of Apoptosis Proteins

Caspases are critical components of both granule-mediated and death receptor-mediated cytotoxicity. Death receptor-induced apoptosis relies on the activation of caspase 8/10 within the DISC to activate the executioner caspases, caspase 3, 6, and 7. Conversely, granzyme B can directly cleave and activate executioner caspases as well as activating them through the mitochondrial pathway of apoptosis. As a result, reducing caspase activity is a common pathway by which cancers avoid apoptosis, with reduced expression or mutations reported for both initiator (caspase 2, 8, and 10) and executioner caspases (caspase 3, 6, and 7) in a range of cancers (191). For example, caspase 8 is commonly mutated, particularly in cancers of neuroendocrine or lymphoid origin (192). Loss of caspase 8 expression contributes to resistance against TRAIL-induced apoptosis (193, 194). In addition to alterations in expression, caspase activity can be modulated by the enhanced expression of IAPs, such as cIAP1, survivin, and X-linked inhibitor of apoptosis protein (XIAP). IAPs can bind directly to caspases preventing their activity and leading to inhibition of granzyme B and death receptor-mediated apoptosis (195–197).

## Bcl-2 Family

The mitochondrial pathway of apoptosis, characterised by mitochondrial outer membrane permeabilization (MOMP), is a critical pathway by which both granzyme B and death receptors can mediate apoptosis. This pathway is regulated by the Bcl-2 family of proteins, which include pro-apoptotic BH3 proteins (e.g. Bid), pro-apoptotic effector proteins (e.g. Bax and Bak), and anti-apoptotic proteins (e.g. Bcl-2 and Bcl-XL) (40). Disruption of this pathway through either downregulation of pro-apoptotic proteins or upregulation of anti-apoptotic proteins can prevent apoptosis induced by either death receptors or granzymes.

A critical mediator of MOMP is Bid, which can be cleaved by either caspase 8 following death receptor ligation or by granzyme B. Bid is responsible for recruiting additional mediators of MOMP, such as Bax and Bak (40). As a result, loss of Bid expression in cancer cells leads to reduced sensitivity to granzyme B-induced apoptosis (198, 199). Likewise, loss of Bak expression, which is directly involved in permeabilization of the mitochondrial membrane, protects against apoptosis triggered by granzyme B (199). Reduced expression of pro-apoptotic Bcl-2 family members, such as Bid, is observed in various cancers and is associated with poor prognosis in prostate cancer and colon cancer, for example (200, 201).

Alternatively, inhibition of MOMP can occur through overexpression of the anti-apoptotic Bcl-2 family proteins, which inhibit the activity of apoptotic proteins, such as Bax and Bak. Overexpression of anti-apoptotic proteins, such as Bcl-2 or Bcl-XL, reduces apoptosis induced by both granule-mediated cytotoxicity and death receptor-mediated cytotoxicity, whereas pharmacological inhibition of Bcl-2 sensitises cells to cytotoxicity (196, 197, 202–206). Overexpression of Bcl-2 is a common feature of multiple cancers (207).

Overall, cancer cells frequently develop mutations or altered expression of the critical caspases and Bcl-2 family members involved in regulating and mediating apoptosis induced by immune attack. As a result, targeting these pathways, for example by inhibition of Bcl-2, could be a particularly effective way of enhancing the efficacy of immunotherapy in patients (208).

## CONCLUSION

Effective cytotoxicity by immune cells against cancerous or infected cells is a critical mechanism of controlling these disease states. A multitude of treatments, such as checkpoint inhibitors, have been developed to boost the immune system's response to these diseased cells. However, these treatments are not always effective, and malignant and infected cells can still exploit mechanisms that enable them to evade the strengthened immune system. Here, we have outlined many ways in which diseased cells can evade the cytotoxic attacks of NK cells and CTLs. Many processes that enhance target cell resistance to cytotoxicity are the same processes that cytotoxic cells themselves use to prevent self-harm by their own deadly cargo.

Other pathways that inhibit cytotoxic killing, such as autophagy, pro-survival TNF signalling, and downregulation of apoptotic pathways, also convey other benefits normally, but can be exploited by cancerous cells or infectious agents.

It is likely that many of these mechanisms of resistance have evolved together with the cytotoxic pathways employed by lymphocytes. This may explain why lymphocytes simultaneously utilise several cytotoxic pathways that are often redundant. For example, the broad range of granzyme B targets – including apoptotic caspases, regulators of mitochondrial apoptosis, and gasdermins – reduces the likelihood that a target cell could become resistant to granzyme B-mediated cell death. This was found to be the case in the context of haematological cancer, in which NK cells and CTLs were able to kill cancer cells despite a variety of anti-apoptotic mutations that conferred multi-drug resistance (209, 210). These studies demonstrate that lymphocytes can overcome some resistance mechanisms by inducing death through multiple pathways.

Conceptually, it can be difficult to distinguish processes that have been autonomously selected to aid the survival of diseased cells that also happen to be beneficial in avoiding immune attack, from processes that have been adopted by these cells to specifically resist cell-mediated cytotoxicity. Further research to investigate this could include studying the evolutionary

development of cancers, and comparisons with mice deficient in specific compartments of their immune system. Either way, there is great potential here to therapeutically target the processes discussed throughout this review. Targeting the ways in which diseased cells avoid death could be used alone or in combination with other therapies, including immunotherapies. Importantly, a greater understanding of these mechanisms and processes *in vivo* is sorely needed to indicate the most potent interventions.

## AUTHOR CONTRIBUTIONS

KT wrote the original manuscript. KT and ARA prepared figures. KT, ARA, and DMD edited the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was funded by Cancer Research UK *via* funding to the Cancer Research UK Manchester Institute (to KT; C147/A25254) and a Wellcome Trust Investigator Award (to DMD; 110091/Z/15/Z).

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DMD receives research funding from GSK, Continuum Life Sciences and Bristol Myers Squibb, and advises GSK, Mogrify and Bicycle Therapeutics.

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