

How Do Cytotoxic Lymphocytes Kill Cancer Cells?

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Abstract

In the past few years, cancer immunotherapy has emerged as a safe and effective alternative for treatment of cancers that do not respond to classical treatments, including those types with high aggressiveness. New immune modulators, such as cytokines, blockers of CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and PD-1 (programmed cell death protein 1)/PD-L1 (programmed death-ligand 1), and interaction or adoptive cell therapy, have been developed and approved to treat solid and hematologic carcinomas. In these scenarios, cytotoxic lymphocytes (CL), mainly cytotoxic T cells (Tc) and natural killer (NK) cells, are ultimately responsible for killing the cancer cells and eradicating the tumor. Extensive studies have been conducted to assess how Tc and NK cells get activated and recognize the cancer cell. In contrast, few studies have focused on the effector molecules used by CLs to kill cancer cells during cancer immunosurveillance and immunotherapy. In this article, the

two main pathways involved in CL-mediated tumor cell death, granule exocytosis (perforin and granzymes) and death ligands, are briefly introduced, followed by a critical discussion of the molecules involved in cell death during cancer immunosurveillance and immunotherapy. This discussion also covers unexpected consequences of proinflammatory and survival effects of granzymes and death ligands and recent experimental evidence indicating that perforin and granzymes of CLs can activate nonapoptotic pathways of cell death, overcoming apoptosis defects and chemoresistance. The consequences of apoptosis versus other modalities of cell death for an effective treatment of cancer by modulating the patient immune system are also briefly discussed. *Clin Cancer Res*; 21(22); 5047–56. ©2015 AACR.

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Introduction

The ultimate goal of the immune response during cancer immunosurveillance and immunotherapy is the elimination of the cancer cells. Cytotoxic lymphocytes (CL), cytotoxic T cells (Tc), and natural killer (NK) cells, are the main players in this process. Other cell types, such as macrophages, mast cells, or dendritic cells, may also kill transformed cells, albeit their specific role and the molecules used for this aim are not clear. Although triggered via distinct receptors, Tc and NK cells use the same basic mechanisms to destroy their target cells: one is elicited by granule exocytosis [i.e., perforin (PRF1) and granule-associated enzymes (granzymes; GZM)], the other via the death ligand/death receptor system (ref. 1; Fig. 1).

Both effector pathways trigger programmed intracellular events in target cells, leading in most cases to apoptotic cell death (2, 3). Accordingly, it has been generally assumed that therapies targeting CLs directly or indirectly would activate those pathways to ultimately kill the cancer cell. However,

looking in detail at the molecular level, it is not so clear which molecules are actually responsible for executing cancer cells during immunosurveillance and immunotherapy. Notably, in some cases, these mechanisms may be different during the native response against cell transformation (i.e., cancer immunosurveillance) and during the elimination of cancer cells by the pharmacologic manipulation of the immune system (see Table 1). Most importantly, under circumstances where apoptosis is blocked by pathogen-derived or endogenous intracellular inhibitors [i.e., inhibitors of apoptosis (IAP) or Bcl-2 (B-cell lymphoma) family members; refs. 3, 4)], CLs are still able to kill cancer cells, indicating that apoptosis is not always required for CL-mediated killing (5–8).

These questions, which may seem trivial for the elimination of cancer cells, are important in the context of recent findings indicating that the subsequent response of the immune system against dying cells greatly depends on the way cells die, that is, if cell death is immunogenic or not (9, 10).

Functioning of Granule Exocytosis and Death Ligands

Stimulation through the T-cell receptor (TCR) or through killer activating receptors (KAR) induces the activation of effector mechanisms by CLs, including expression and release of death ligands like FasL (Fas ligand) and TRAIL (TNF-related apoptosis inducing ligand; refs. 11–13) and the granule exocytosis pathway (refs. 14, 15; Fig. 1). The granule exocytosis pathway is rapidly executed by a directional mobilization of preformed specialized cytoplasmic granules, toward the contact site of CLs and target cells (the immunological synapse), where their content is released (14, 15). The pore forming protein, PRF1 (16), along with GZMs, which are members of a family of serine proteases, are the dominant constituents of the

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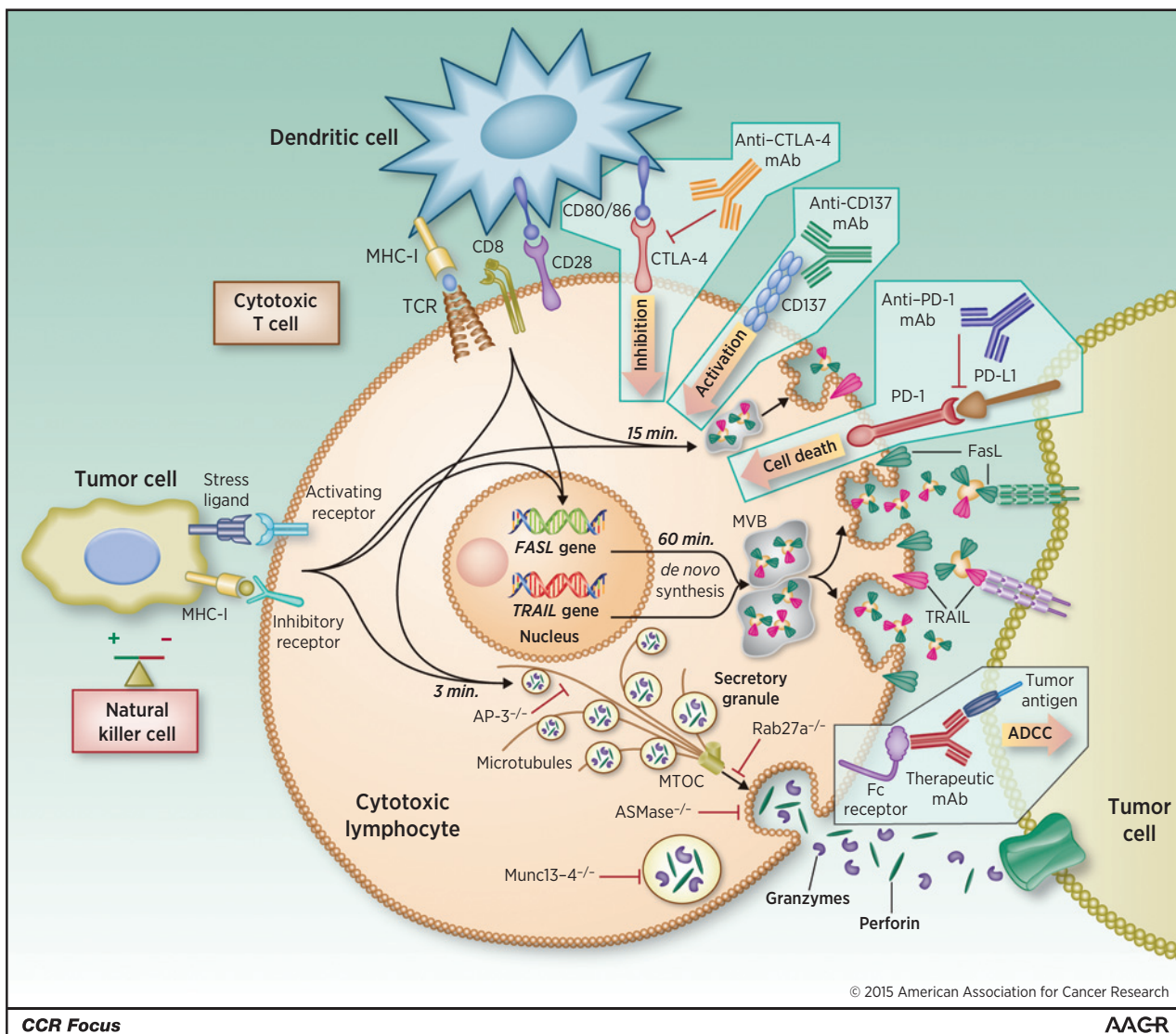


Figure 1. Activation of the main effector mechanisms of cytotoxic lymphocytes. Activation of cytotoxic T cells (Tc) is an antigen-specific process requiring the interaction of the TCR-CD3 complex with a processed tumor antigen-derived peptide bound to a MHC class I molecule as well as costimulatory signals (CD8 and CD28). Activation of NK cells (NK) relies on the balance between activating and inhibitory receptors provided by tumor cells (left). Although the mechanisms of activation of Tcs and NKs are quite different, both cytotoxic lymphocytes (CL) share common effector mechanisms: granule exocytosis and the death ligand/death receptor system. Upon CL activation, the microtubule-organizing center (MTOC) rapidly polarizes the traffic of preformed secretory granules toward the presynaptic membrane (middle). The secretory granules then fuse with the plasma membrane at the immunological synapse and release their content (perforin and granzymes), leading to tumor elimination. Deficiency in proteins controlling intracellular trafficking and granule fusion and release affects exocytosis at different levels, reducing the ability of CLs to kill target cells (15). During death ligand/death receptor-mediated apoptosis, upon CL reactivation, preformed FasL (Fas ligand) and TRAIL are expressed on the surface of CLs or released as exosome membrane-bound death ligands after fusion of multivesicular bodies (MVB) with the cell-cell contact zone. Reactivation of CLs also induces FasL and TRAIL *de novo* synthesis, leading to formation of new death ligand-associated exosomes and increasing death ligand surface expression. FasL and TRAIL expressed and released from CLs are able to kill susceptible tumor cells through their interaction with their respective death receptors. Activation of these effector mechanisms can be modulated by several monoclonal antibodies (mAb). Immunomodulatory activity thus enhances antitumor activity of CLs, and mAbs can bind to Fc receptors expressed by NK cells, allowing antibody-dependent cell cytotoxicity (ADCC). Blocking mAbs against immune checkpoint molecules (CTLA-4 and PD-1) prevents, respectively, inhibitory signals or cell death signals that CLs receive from these molecules. Finally, agonistic mAbs against costimulatory molecules such as CD137 lead to the increase of CL cytotoxic activity against tumor cells (right).

cytolytic granules (7, 8). GZMA and GZMB have attracted most of the attention over the past few decades. However, additional GZMs with possible functional significance (in total 5 in humans and 10 in mice) and other cytoplasmic granule-

associated molecules like the human-specific protein granzulin (17) have been described, though their biologic functions during cancer immunity and immunotherapy have not been elucidated (5, 7, 18).

Table 1. Cancer susceptibility of mice deficient in the main cell death effectors of CL

Mouse genotype	Immunosurveillance ^a	Immunotherapy ^c				
		Cytokines	CTLA-4 PD-1/PD-L1	ACT	CD137	
Prf ^{-/-}	MCA-induced sarcoma ^b (31)	IL12 melanoma, sarcoma, ^f		RKIK sarcoma ^f (76)	EL4 lymphoma ^f (65)	
	Spontaneous B-cell lymphoma ^b (34, 35)	(66, 67, 69, 70, 72)		B16 melanoma ^e (74, 75)		
	Oncogene (TP53, v-Abl, Bcl-2, Mlh-1)-driven B-cell lymphoma ^b (35)	IL15 ^f (68)		Renal carcinoma ^e (81)		
	HER2/neu-driven breast carcinoma ^b (37, 38)	IL12 renal ^e (60)	αGalCer (alpha-galactosylceramide) renal, melanoma ^e (60, 71)	Prostate, colorectal, and breast carcinoma ^e (63)		
GzmA ^{-/-}	MCA-induced sarcoma ^d (31)			RKIK sarcoma ^e (76)		
GzmB ^{-/-}	MCA-induced sarcoma ^d (31)			RKIK sarcoma ^e (76)		
GzmAxB ^{-/-}	MCA-induced sarcoma ^d (31)					
GzmM ^{-/-}				RKIK sarcoma ^f (76)		
TRAIL ^{-/-}	MCA-induced sarcoma ^b (54–56)	αGalCer (anti-TRAIL ab) ^f (60)		RKIK sarcoma ^f (76)		
	Spontaneous B lymphoma ^b (58)			RKIK sarcoma ^f (76)		
	Oncogene (TP53)-driven B-cell lymphoma ^b (58)					
	HER2/neu-driven breast carcinoma ^d (58)					
TRAIL-R ^{-/-}	Oncogene (Eμ-Myc)-driven B-cell lymphoma and associated lung metastasis ^b (56)					
	DEN-induced hepatocarcinoma ^b (56)					
	Radiation-induced T lymphoma ^b (56)					
	DMBA/TPA-induced primary squamous cell carcinoma ^d (62)					
	Metastasis during DMBA/TPA-induced squamous cell carcinoma ^b (62)					
	Lpr ^g	Spontaneous B-cell lymphoma ^b (48)				
		Spontaneous plasmacytoid tumors ^b (49)				
FasL ^{-/-}				B-cell lymphoma ^f (47)		
Gld ^g	Spontaneous plasmacytoma ^b			RKIK sarcoma ^e (76)	EL4 lymphoma ^f (65)	

^aSusceptibility of the corresponding mouse strain to chemical, spontaneous, and oncogene-driven carcinogenesis.

^bIncreased susceptibility compared with wild-type mice.

^cEfficacy of different immunotherapy protocols in the corresponding mouse strains.

^dSame susceptibility as wild-type mice.

^eTreatment is as efficient in knockout mice as in wild-type mice.

^fTreatment is less efficient in knockout mice than in wild-type mice.

^gGld and Lpr are mouse strains with natural mutations for FasL and Fas, respectively.

In most cases, PRF1 acts as a vehicle for the delivery of GZMs into the cytosol of the target cell by a mechanism that seems to be dependent on its ability to form pores in membranes (16). Paradoxically, this event, one of the most critical steps controlling the elimination of cancer cells, is still a matter of intense debate that is only now beginning to be clarified. It seems that, as suggested almost 30 years ago, PRF1 forms pores in the plasma membrane to allow GZMs to access the target cell cytosol, although the nature of the pore is not clear (19, 20). However, it is still unknown if the alternative models proposed (receptor- or clathrin-mediated GZM endocytosis and release from endosomes by coendocytosed PRF1) operate under some circumstances depending on the target cell (21). In addition, when using susceptible target cells or in specific situations where GZMs would not be expressed or would be inhibited, PRF1 *per se* may be able to kill target cells by inducing cell lysis. This hypothesis is supported by experiments showing that rat basophil leukemia cells transfected with PRF1 cDNA lyse Jurkat cells (22). In this context,

changes in the lipid composition of the plasma membrane in cancer cells may influence its response (either as GZM delivery or as a lytic agent) to PRF1, modulating the sensitivity of cells to CLs and immunotherapy (23, 24).

Once released in the cytosol, GZMs would execute the target cells by cleaving critical intracellular substrates controlling cell death and survival. Substrates of GZMs also include viral and cellular proteins crucial for virus replication (25) as well as extracellular matrix proteins controlling vascular integrity, inflammation, and skin aging (26–28) but this will not be treated in this article. However, which GZMs activate cell death and the features of dying cells are only now beginning to be clarified in physiologic models.

Death ligands are proteins expressed by CLs that bind the members of the TNF superfamily with ability to trigger target cell death (death receptors). Among the known death ligands, Tcs and NK cells mostly express TNFα, FasL, and TRAIL, which can be expressed at the membrane of the CL or secreted to exosomes

(Fig. 1). The main role of FasL and TRAIL seems to be associated with the control of T-cell homeostasis by a process known as activation-induced cell death. Although all these proteins are able to induce cell death in susceptible target cells when used in purified form their contribution to CL-mediated cell death and tumor immunosurveillance is less clear, as discussed below. As summarized in Fig. 2, the main cell death form triggered by death ligands in target cells is eminently apoptotic, involving the activation of the extrinsic and the intrinsic or mitochondrial pathways depending on the cell type. However, in specific target cells, and depending on the expression of intracellular inhibitors, death ligands could perform different functions, including induction of other types of cell death or even contributing to tumor cell survival and proliferation. These contrasting effects are addressed in more detail below.

Who Is Who during Cell Death Induced by CLs in Cancer Immunoreveillance and Immunotherapy?

Cancer immunoreveillance

Most of the evidence gained from studies using mouse *in vivo* models indicate that PRF1 is a key factor for NK- and Tc-mediated control of both transplanted syngeneic tumors as well as during chemical carcinogenesis (Table 1; and refs. 2, 29–31). This also applies to control of cancer metastasis (2, 32). Indeed, early as well as more recent studies indicate that Tc and NK cells from PRF1-deficient mice present a great impairment to fast and efficiently induce cell death on most target cells (33). The role of PRF1-mediated cell death in cancer immunoreveillance has been clearly established during spontaneous cancer development (Table 1). This seems to be specially relevant for tumors of hematologic origin as PRF1 knockout mice develop spontaneous B lymphoma (34). In addition, PRF1 deficiency enhances the oncogenic potential of diverse proteins such Abl-1 (Abelson murine leukemia viral oncogene homolog 1), Bcl-2 or Mlh1 (MutL homolog 1; ref. 35). Importantly, PRF1 deficiency in humans seems to predispose to development of several types of lymphoma and leukemia (36). Concerning other types of tumors, it has been recently shown that PRF1 deficiency accelerates the onset of HER2/neu-driven breast carcinomas (37, 38) as well as neoplastic grading (38).

Concerning the role of GZMs in cancer immunoreveillance the results are less clear and a consensus has not been reached. Some groups have reported that mice deficient in GZMA and GZMB present a higher susceptibility to NK cell-sensitive implanted cancer cell lines (30, 39, 40). In contrast, others have reported that mice deficient in these GZMs control implanted tumors as well as chemically induced sarcomas as efficient as wild-type mice (31, 41). Here it should be noted that in some models GZMB deficiency abrogates the function of CD4⁺ T regulatory cells, increasing the antitumor response of CLs (ref. 42; and J. Pardo; unpublished data), which may mask the antitumor potential of CL-associated GZMB.

Although these discrepancies have not been clarified yet, it has been argued that other GZMs might compensate for the absence of GZMA and GZMB; meanwhile, PRF1 deficiency would inactivate the antitumor function of all GZMs. However, deficiency in other GZMs, such as GZMM (43), does not increase the susceptibility to implanted syngeneic cancer cell

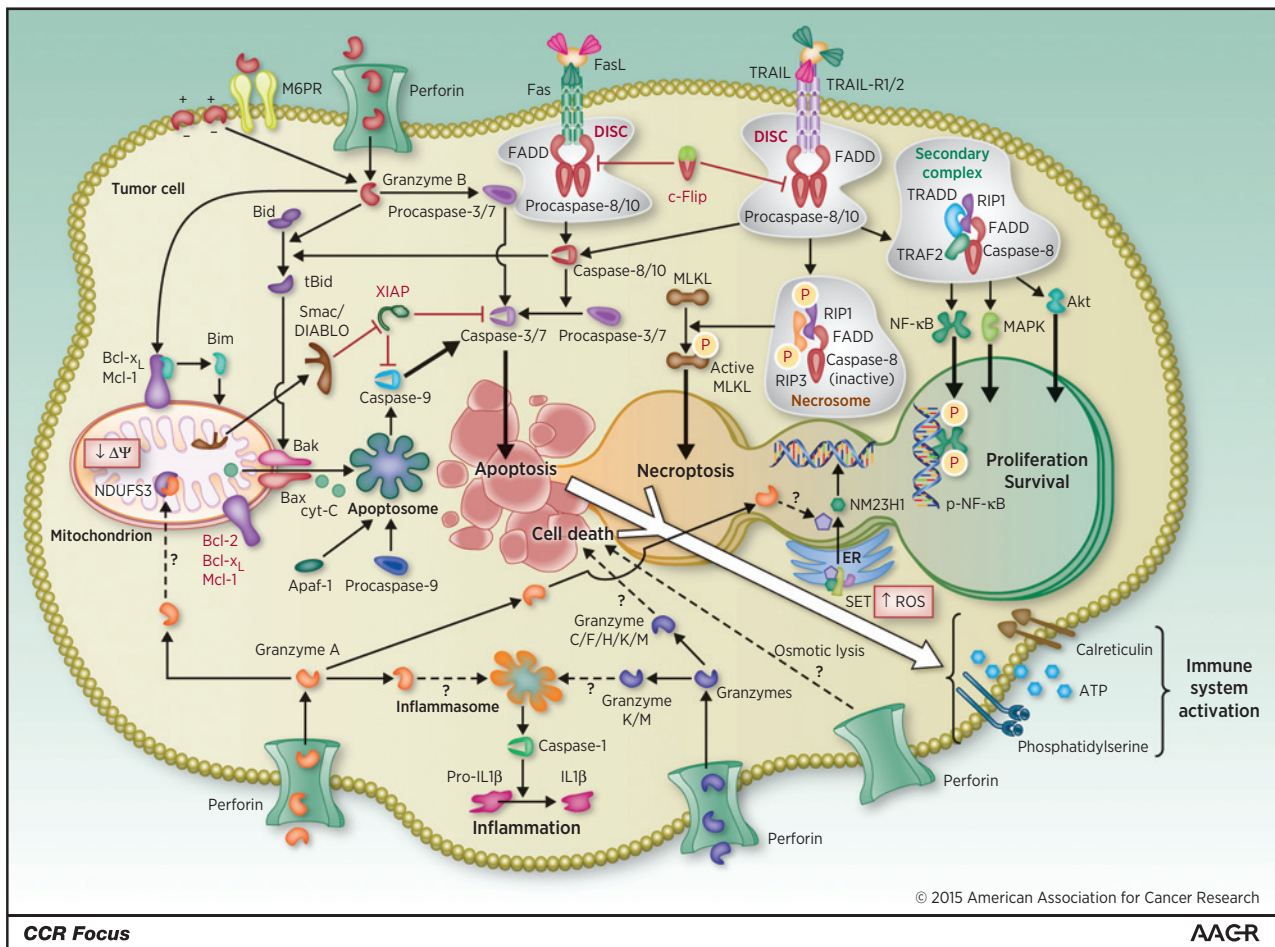
lines, including lymphoma and melanoma. We still do not know the phenotype of mice deficient in GZMK but it should be expected that their susceptibility to tumors is not increased as its cytotoxic potential *in vitro* is very low (44). It is possible that, in the absence of GZMs, PRF1 *per se* would eliminate cancer cells by inducing cell lysis in a similar way to complement membrane attack complex as previously suggested (ref. 16; Fig. 2).

In some cases, cell death induced by PRF1-deficient CLs can be restored at longer incubation times in specific tumor cell types that present sensitivity to FasL, suggesting that this death ligand may also contribute to tumor immunoreveillance *in vivo* (12, 45, 46). In fact, it was recently shown that Tc cells use FasL to eradicate transplanted B-cell lymphoma cells in RAG1 (recombination activating gene 1)-deficient mice (47). This result confirms previous findings using animals with natural mutations in Fas (lpr) or FasL (gld; refs. 48, 49). Supporting these studies, the presence of Fas mutations in human lymphoproliferative disorders was correlated with a higher incidence of B and T lymphoma (50, 51). However, in this case, development of lymphoma could be related to a defect in activation-induced cell death rather than FasL-mediated immunoreveillance.

The other major death ligand, TRAIL, was first described as a cytokine capable of inducing apoptosis in a wide variety of cancer cells while sparing normal cells. However, its main role seems to be regulation of the immune response (52, 53). The role of endogenous TRAIL in tumor immunoreveillance is not fully understood yet. A few *in vitro* studies have clearly shown that NK cells are able to kill cancer cells using TRAIL (11, 54). Indeed, it seems that TRAIL is a mechanism used by liver but not spleen NK cells to prevent tumor metastasis. As shown in Table 1, it has been found that TRAIL- or TRAIL-R (TRAIL-receptor)-deficient mice are more susceptible to some transplanted tumors (55) as well as chemical carcinogenesis (54, 56). Animals deficient in TRAIL and TRAIL-R do not spontaneously develop tumors at an early age (54, 57), but aged TRAIL-deficient mice present a slightly increased susceptibility to develop spontaneous lymphoma (58). This susceptibility seems to be more pronounced in the context of loss of at least one allele of p53 (11, 58). In contrast, other groups found that development of spontaneous tumors was not increased in TRAIL-R-deficient mice in the context of p53 loss (59). Despite these contradictory findings in the control of primary tumors, it is commonly accepted that TRAIL is involved in cancer immunoreveillance in controlling tumor metastasis. This effect was originally shown using transplanted syngeneic cell lines (11, 54, 60, 61) and later confirmed in a more physiologic model of spontaneous metastasis during chemically induced carcinomas (62). In contrast to PRF1 and Fas, no human mutations in TRAIL or TRAIL-R have been described that correlate with a higher predisposition to cancer development.

Cancer immunotherapy

In contrast to developments in cancer immunoreveillance, the involvement of PRF1 and GZMs during cancer immunotherapy has not been explored in depth, and the results to date are not clear. The potent immunodominant Tc-cell epitope, lymphocytic choriomeningitis virus (LCMV) peptide gp33, has been widely used as a model tumor antigen to analyze

**Figure 2.**

Effector mechanisms of cytotoxic lymphocytes on tumor cells. Perforin-delivered intracellular granzyme B is capable of inducing cell death by different pathways. Granzyme B can directly cleave and activate the effector caspase-3 and -7. On the other hand, granzyme B also cleaves the proapoptotic Bcl-2 (B-cell lymphoma 2) family protein Bid, generating truncated Bid (tBid), which in turn activates Bak (Bcl-2 homologous antagonist killer)/Bax (Bcl-2-associated X protein) oligomerization on the mitochondrial outer membrane, allowing the release of cytochrome C (cyt C) from mitochondria. Once in the cytoplasm, cytochrome C, apoptotic protease activating factor 1 (Apaf-1), and procaspase-9 form a multimolecular complex called an "apoptosome," in which caspase-9 is activated. In parallel, release of Smac/DIABLO (Second mitochondria-derived activator of caspases/Direct IAP-binding protein with low PI) prevents the inhibitory function of X chromosome-linked inhibitor of apoptosis (XIAP), thereby allowing caspase activation. Finally, granzyme B can also activate the mitochondrial pathway by inducing the delivery of the proapoptotic Bcl-2 family protein Bim from its association with antiapoptotic proteins Mcl-1 (myeloid cell leukemia-1) and Bcl-x_L (B-cell lymphoma-extra large). Granzyme A regulates the production of proinflammatory cytokines (IL1 β) by a mechanism dependent on caspase-1. The contribution of the inflammasome platforms to this process is suggested although not proven yet. *In vitro* experiments using purified proteins, it has been described that granzyme A is also able to cleave a protein known as NADH dehydrogenase ubiquinone iron-sulfur protein 3 (NDUFS3) inducing mitochondrial depolarization ($\downarrow \Delta\Psi$) and reactive oxygen species (ROS) production. ROS generation in turn induces DNA damage and the subsequent activation of DNA-repairing mechanisms, among them, the SET complex, which translocates from the endoplasmic reticulum (ER) to the nucleus. In the nucleus, granzyme A would cleave some SET complex proteins such as SET, pp32, and Ape1 (apurinic/apyrimidinic endonuclease 1) releasing the nuclease NM23H1 (nonmetastatic clone 23 human 1). In turn, released NM23H1 would induce DNA damage triggering cell death. It has been described that purified granzymes C, F, H, K, and M are able to induce cell death in the presence of perforin by activating diverse intracellular pathways, although the physiologic relevance of this ability has been questioned (5, 7, 79). In addition, it has been reported that granzymes K and M can regulate the production of proinflammatory cytokines. Induction of cell death of tumor cells by CLs induces phosphatidylserine translocation of calreticulin and maybe other danger signals such as adenosine triphosphate (ATP). These events are necessary for a proper activation of the immune system against the dying tumor cells. Regarding death ligands, FasL (Fas ligand) and TRAIL (TNF-related apoptosis inducing ligand) bind to their respective death receptors, Fas for FasL and TRAIL-R1/2 for TRAIL, promoting receptor oligomerization. Consequently, the oligomerized death receptors recruit the adaptor protein Fas-associated death domain (FADD) through homotypic interaction between their death domains. The death effector domain of FADD in turn binds procaspase-8, allowing its transactivation. Active caspase-8 triggers two different apoptotic pathways depending on the cell type. Active caspase-8 cleaves procaspase-3, which is able to degrade distinct substrates leading to cell death by apoptosis and also the BH3-only proapoptotic protein Bid, generating tBid, which, as described above, activates the mitochondrial apoptotic pathway. Apoptosis through the death receptor pathway can be inhibited at different levels. Cellular FLICE inhibitory protein (c-FLIP) can compete with caspase-8 for the binding to FADD inhibiting caspase-8 activation. In some circumstances in which caspase-8 is inactive, TRAIL-Rs and possibly also Fas ligation can recruit receptor interacting protein (RIP1) and RIP3, forming a complex called a "necrosome," which phosphorylates MLKL (mixed lineage kinase domain-like protein), promoting its oligomerization. Then, MLKL inserts into and permeabilizes plasma membrane leading to necrotic cell death. Finally, TRAIL can also trigger proliferation and survival signals if apoptosis is blocked. TRAIL-Rs also can recruit RIP upon TRAIL binding, leading to a secondary complex formation containing TNF receptor-associated factor 2 (TRAF2) and TNF receptor type 1-associated death domain (TRADD). RIP1 can then promote the activation of the transcription factor NF- κ B and of MAPK and Akt kinase (protein kinase B), promoting survival signals.

the elimination of cancer cells by activating virus-specific Tc-cell responses. Using this model, we have found that prevention and elimination of syngeneic grafted cancer cell lines of diverse origin are dependent upon the presence of PRF1 (J. Pardo; manuscript under preparation).

Several investigations have been pursued to reveal the role of PRF1, GZMs, and death ligands during the elimination of cancer cells by immune modulators used in clinics, including immunostimulatory antibodies and cytokines (Table 1). However, the results are difficult to interpret since generally different tumor models have been used. A summary of these results is depicted in Table 1. Results are indicated as the efficacy of every treatment in PRF1, GZMs, and death receptors/death ligand-deficient mice compared with wild-type mice.

In vivo elimination of colon, prostate, and breast carcinoma cell lines mediated by anti-CD73, anti-CTLA-4, anti-PD-1 mAbs or the combination of them is not affected by the absence of PRF1 (63). In contrast, PRF1 was shown to contribute significantly to the antitumor effect of the combination of BRAF (B-Raf proto-oncogene, serine/threonine kinase) inhibitors and agonistic anti-CD137 antibody in melanoma cells (Table 1; ref. 64). We have recently found that anti-CD137-mediated elimination of EL4 lymphoma in mice depends on both PRF1 and FasL (65). More experimental evidence will be required to find out whether the contribution of PRF1 in mAb-mediated control of tumors is dictated by the type of cancer cell and/or by the type of stimuli.

Regarding cytokine therapy, most studies have focused on cytokines that predominantly activate NK/NKT cell-mediated responses. The elimination of melanoma and sarcoma murine cell lines by IL12 (66, 67) or IL15 (68) was found to be dependent on PRF1 expression. PRF1 was also required to control melanoma tumor metastasis by IL12 (69, 70). In contrast, *PRF1* deficiency did not affect IL12 and α GalCer (alpha-galactosylceramide)-mediated control of liver metastasis using the RENCA renal carcinoma model. Another study confirmed that *PRF1* deficiency did not affect the antimetastatic activity of α GalCer in the B16 melanoma model (71). Finally, it was shown that the antitumor effect of IL12 against melanoma cells in mice was dependent on PRF1 (72). As previously suggested, it seems that the effect of IL12 during cancer immunotherapy is model dependent (73).

Another approach used to treat cancer is adoptive cell transfer (ACT), which consists of the administration to the cancer-bearing host of Tc or NK cells with direct anticancer activity. Some researchers have also analyzed the effector molecules of CLs involved in cancer elimination during ACT. These studies revealed that Tcs or NK cells from *PRF1*-deficient mice are as efficient as wild-type cells in controlling lung metastasis in the B16 melanoma model (74, 75). A recent study by Pegram and colleagues shows that PRF1, GZMB, and GZMM are required to inhibit the growth of a transplanted sarcoma cell line during adoptive NK-cell transfer (76). In contrast, the absence of GZMA did not affect tumor growth. The lack of antitumor activity of GZMA in this model supports more recent findings questioning the cytotoxic potential of this and other GZMs (77–79).

Concerning the role of death ligands during immunotherapy using the gp33 antigen tumor model, we found that prevention and elimination of syngeneic grafted cancer cell lines of diverse origin is not affected by FasL deficiency (J. Pardo; manuscript in preparation). The efficacy of different immunotherapy

approaches in death receptor/death ligand-deficient mice is summarized in Table 1. FasL or TRAIL did not contribute significantly to the antitumor effect of the combination of BRAF (B-Raf proto-oncogene, serine/threonine kinase) inhibitors and agonistic anti-CD137 antibody in melanoma cells (Table 1; ref. 64). In contrast, as indicated above, both PRF1 and FasL cooperated during anti-CD137-mediated elimination of EL4 lymphoma (65).

FasL has also been involved in IL18-mediated elimination of B16 melanoma cells (72). Anti-TRAIL mAb therapy blocked IL12 and α GalCer-mediated control of liver metastasis using the RENCA renal carcinoma model, indicating a critical role of TRAIL in this protective effect (60). Finally, in a model of ACT, TRAIL was also found to be required for the antitumor function of transferred NK cells against a sarcoma cell line (76).

A conclusion that can be reached from these studies is that in some types of cancer the cell death executors involved in cancer immunosurveillance may be different from those activated by immunotherapy. This hypothesis is strongly supported when comparing the studies that analyze immunosurveillance and immunotherapy using similar tumor models (Table 1), in which it has been found that, *GZMB*-deficient mice are not more susceptible than wild-type mice to sarcomas induced by MCA (31), but they are compromised in the control of implanted sarcomas during adoptive NK-cell transfer (76). *PRF1*-deficient mice are more susceptible than wild-type mice to oncogene-driven or to implanted mammary carcinomas (32, 37, 38). In contrast, they control implanted mammary carcinoma cells during mAb therapy as efficiently as wild-type mice (63). *PRF1* deficiency increases the susceptibility to liver and lung metastasis in the RENCA renal carcinoma model (80) but does not affect the control of metastasis during IL12/ α GalCer (60) or adoptive Tc-cell therapy (81). *PRF1* deficiency increases the susceptibility to the implanted prostate cancer cell line RM1 (32), but this deficiency has no impact on mAb-mediated control of this cell line (63).

The differences observed during the elimination of cancer cells in immunosurveillance and/or immunotherapy could be related to the strength of the stimuli recognized by CLs, as recently suggested (82).

Who Makes What during Cell Death Induced by CLs?

Apoptotic and nonapoptotic pathways activated by granule exocytosis

It has been assumed that the final consequence of the concerted action of PRF1 and GZMs is the induction of cell death by a mechanism known as apoptosis (ref. 5; Fig. 2). However, this overreaching conclusion mainly obtained from *in vitro* models using purified GZMs delivered with a great variety of agents may not be justified. In contrast, recent evidence indicates that neither all GZMs present cytotoxic potential nor is the mechanism of cell death activated by CL always apoptosis (refs. 7, 79; Fig. 2).

First of all, to properly understand some of the results obtained using CLs from *GZM*-deficient mice, it is worth mentioning again that PRF1 alone may be able to lyse specific target cells under the circumstances mentioned above. This effect has only been shown in Jurkat cells used as effector cells in rat basophil leukemia cells transfected with *PRF1* cDNA, and it should be confirmed using CLs. PRF1 lytic activity could be dependent on the amount of PRF1 delivered by the effector cell, the susceptibility of the target

cell membrane, and/or the ability of the target cell to repair the PRF1 pores.

In this context it has been shown that antigen-specific Tcs and NK cells from GZMA and GZMB double-knockout mice still present some ability to induce cell death on tumor target cells *ex vivo* (31, 83, 84) as well as during *in vivo* peritoneal killing (85), although at a reduced level in comparison with CLs from wild-type mice. However this type of cell death does not present clear apoptotic features and proceeds with membrane permeabilization in the absence of caspase activation (84, 86). Notably, it was later shown that cells eliminated under these circumstances were not efficiently phagocytosed by DC cells and did not induce antigen cross-presentation (85). Supporting this finding, we found that GZMB was required for immunogenic calreticulin exposure in plasma membrane of the dying cells during Tc-cell attack (87).

Several GZMs, including human and mouse GZMA, GZMB, GZMK, and GZMM, as well as human GZMH or mouse GZMC, have been shown to induce cell death *in vitro* by using purified molecules (6, 8, 18). Excluding GZMB that clearly activates apoptosis involving both caspases (3) as well as the mitochondrial intrinsic pathway regulated by the Bcl-2 family (4, 6), the molecular mechanisms of cell death activated by purified GZMs are not apoptotic (6, 18) and are often contradictory (ref. 88; Fig. 2). However, this will not be the focus of our discussion, as it has been reviewed elsewhere (6). Moreover, it is not clear whether all GZMs are indeed inducing cell death when delivered by CLs. Indeed, CLs from GZMM or GZMA knockout mice do not present any defect to kill most target cells. Recent evidence from several independent groups combining data generated from purified molecules and CLs has confirmed that the cytotoxic potential of human GZMA is very low if it exists at all (77–79, 89). In mice, it has been observed that GZMA may induce cell death in specific cancer cell lines (90) by a mechanism that requires an intact actin cytoskeleton. This process does not resemble all features of apoptosis and has been named "athetosis" (91).

As mentioned above, the ability of GZMs to induce apoptosis during CL attack has been confirmed only for GZMB (7). CLs from mice deficient in the GZMB cluster are unable to induce fast oligonucleosomal DNA fragmentation (92) and phosphatidylserine (PS) translocation in the absence of membrane permeability (93). Indeed, GZMB has been shown to be crucial for CL-mediated caspase-3 and Bid activation (93) and degradation of specific intracellular substrates such as tubulin (94, 95), Mcl-1 (myeloid cell leukemia-1), or Bcl-x_L (B-cell lymphoma-extra large; ref. 96). Importantly, these events occurred before membrane permeabilization could be detected (Fig. 2). Notably, the mechanism of cell death activated by GZMB maybe dependent on the species (78) as well as on the type of cell transformation (96). In humans, it has been shown that cell cytotoxicity of cytokine-activated human NK cells is greatly reduced by inhibiting GZMB (97, 98), confirming that GZMB is the main cell death inductor in CLs. To further prove that cell death induced by GZMB is important during Tc cell-mediated cancer immunotherapy we used the LCMV gp33 antigen model. Here, we found that Tc cells require GZMB-mediated cell death to prevent development of tumors at long term (J. Pardo; manuscript in preparation).

However, cell death induced by CLs through GZMB is not always apoptotic in nature. This fact is particularly evident when target cells in which apoptotic pathways are blocked are

used. It has been found that Tc cells use PRF1 and GZMB to kill cells in which both the intrinsic mitochondrial pathway and caspases are blocked (93), highlighting the potential benefits of immunotherapy to treat cancer cells that do not respond to conventional therapy (3, 4). We have recently confirmed in humans that allogeneic activated NK cells expressing GZMB eliminate hematologic cancer cells in which apoptosis is blocked by p53 mutation and overexpression of Bcl-x_L or downregulation of Bak and Bax even in the presence of caspase inhibitors (99). However, under these circumstances, the phenotype of dying cells is not apoptotic, and PS translocation did not precede membrane permeability. The characteristics of this type of cell death as well as its consequences for the immune system are currently being explored.

In conclusion, cell death induced by CLs in the absence of GZMB or in target cells in which apoptosis is blocked may not be enough to amplify the antitumor immune response and establish antitumor memory that prevents a future tumor relapse.

Other modalities of cell death

As indicated above, granule exocytosis can induce cell death independent of apoptosis. At present, it is unknown whether other mechanism of cell death and/or survival such pyroptosis, necroptosis (3), or autophagy (100) may regulate cell death executed by CL. During the last years, it has been found that some GZMs like GZMA, GZMK, and GZMM present inflammatory potential and may regulate the production of IL1 β , TNF α , and IL6 by macrophages in a caspase-1-dependent manner (refs. 77, 101; Fig. 2). Indeed, GZMA-deficient mice resist sepsis without compromising other protective functions like Tc cell-mediated elimination of infected macrophages (102). However, caspase-1 activation does not lead to macrophage cell death in these conditions as in the case of pyroptosis induced by bacterial infection. Alternatively, it could be that inflammation induced by those GZMS (7, 101, 89) as well as the reported effects of GZMB on extracellular matrix degradation and inflammation (27, 89) could either enhance the antitumoral response of the immune system or be detrimental during development of inflammatory carcinomas. Certainly, this interesting novel aspect of the biology of granule exocytosis will be the focus of upcoming studies in cancer immunosurveillance and immunotherapy.

Death ligands

It has been known for some time that death receptor ligation leads to caspase-dependent apoptotic cell death (ref. 3; Fig. 2). However, the mechanism of cell death shifts from apoptosis to necroptosis in the presence of caspase inhibitors (ref. 103; Fig. 2). The molecular mechanism of death receptor-induced necroptosis, which involves the kinases RIP1 (receptor-interacting protein 1) and RIP3, has been described recently (103). The possibility that tumor cells resistant to death receptor-induced apoptosis could shift their mode of cell death toward necroptosis could have an impact on immunogenicity and the subsequent action of immune surveillance mechanisms as well as on the efficacy and side effects of immunotherapy treatments.

On the other hand, TRAIL also regulates proinflammatory responses through activation of the NF- κ B pathway (ref. 53; Fig. 2). This characteristic could be exploited by tumor cells for their own benefit promoting proliferation, migration, and invasion of cancer cells (104, 105). Indeed, in a pancreatic

adenocarcinoma xenograft model, it has been described that tumor cells used TRAIL to promote the development of metastases in the liver (105). In this cell line, it was found that FasL also enhances motility and invasiveness in a variety of apoptosis resistance cancer cells (106). More recently, it was shown that signaling through TRAIL receptors can be used by tumor cells to promote KRAS-driven tumorigenesis (107).

Concluding Remarks

CLs (Tc and NK cells) are the main effector cells executing transformed cells during cancer immunosurveillance and immunotherapy. However, the experimental evidence suggests that the molecular mechanisms involved in immunosurveillance are not always the same as those in immunotherapy. PRF1/GZMB is the most potent pathway used by CLs to kill cancer cells, overcoming antiapoptotic mutations, including p53 deletion/mutation, overexpression or downregulation of members of the Bcl-2 family, and caspase inhibition. Thus, under these circumstances, apoptosis is not required for CL-mediated target cell killing. Notably, in the absence of GZMB (i.e., gene mutation or expression of endogenous inhibitors), PRF1 *per se* could induce cell lysis in susceptible target cells. In contrast, TRAIL seems to be involved in the control of metastasis and FasL could compensate in some instances of PRF1 deficiency. Originally the main effector pathways of CLs, PRF1/GZMs and death ligands, were thought to act exclusively by inducing apoptotic cell death on transformed cells. Recent experimental evidence indicates that during the interaction between CLs and tumor cells, nonapoptotic cell death pathways,

inflammation induced by some granzymes and death ligands, and proliferative effects of death ligands may unexpectedly contribute to cancer progression rather than control. A better understanding of how CLs actually kill cancer cells during immunotherapy will help to predict patient responses and to select the best protocols to obtain activated CLs that efficiently kill tumor cells without inducing other undesirable effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Martinez-Lostao, A. Anel, J. Pardo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Pardo
Writing, review, and/or revision of the manuscript: L. Martinez-Lostao, A. Anel, J. Pardo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Martinez-Lostao, J. Pardo

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