How do cytotoxic lymphocytes kill cancer cells?

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Summary

During the last years cancer immunotherapy has emerged as a safe and effective alternative to cancers that do not respond to classical treatments including those types with high aggressiveness. New immune modulators like 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) interaction or adoptive cell therapy have been developed and approved to treat solid and haematological carcinomas. In these scenarios, cytotoxic lymphocytes (CL), mainly cytotoxic T (Tc) and Natural Killer (NK) cells are the ultimate responsible of killing the cancer cells and eradicate the tumour. Extensive studies have been developed on how Tc and NK cells get activated and recognise the cancer cell. In contrast few studies have been focused on the effector molecules employed by CL to kill cancer cells during both cancer immunosurveillance and immunotherapy. During the next sections the two main pathways involved in CL-mediated tumour cell death, granule exocytosis (perforin and granzymes) and death ligands, will be briefly introduced followed by a critical discussion of the molecules involved in cell death during cancer immunosurveillance and immunotherapy. Recent experimental evidences indicate that perforin and granzymes of CLs can activate non-apoptotic pathways of cell death overcoming cellular resistance to traditional treatments. In addition, neither all granzymes present cytotoxic potential nor death ligands always contribute to cancer elimination. Here the consequences of apoptosis versus other modalities of cell death for an effective treatment of cancer by modulating the patient immune system will be also briefly discussed.

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 (Tc) and Natural Killer (NK) cells, are the main players in this process. Other cell types like macrophages, mast cells or dendritic cells may also kill transformed cells albeit its specific role and the molecules used for this aim are not clear. Although triggered via distinct receptors, Tc and NK cells employ the same basic mechanisms to destroy their target cells: one is elicited by granule exocytosis (i.e. perforin –PRF1- and granzymes – GZM-), the other via the death ligand/death receptor system (Fig. 1) {Russell, 2002 #158}.

Both effector pathways trigger programmed intracellular events in target cells, leading in most cases to apoptotic cell death {Bolitho, 2007 #333; Fulda, 2015 #3006}. Accordingly, it has been generally assumed that therapies targeting direct or indirectly CL would activate those pathways to ultimately kill the cancer cell. However, looking in the detail at the molecular level one realises that it is not so clear which molecules are the actual responsible of executing cancer cells during both 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 pharmacological manipulation of the immune response (see Table 1). Most importantly, under circumstances where apoptosis is blocked by pathogen-derived or endogenous intracellular inhibitors (i.e. IAPs or Bcl-2 family members) {Gibson, 2015 #3008; Fulda, 2015 #3006}, CL are still able to kill cancer cells, indicating that

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apoptosis is not always required for CL-mediated killing {Voskoboinik, 2015 #2958; Ewen, 2011 #2925; Pardo, 2009 #385; Chowdhury, 2008 #1205},.

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 how cells die i.e. if cell death is immunogenic or not {Zitvogel, 2010 #2893; Casares, 2005 #2990}.

Functioning of granule exocytosis and death ligands.

Stimulation through the TCR or through killer activating receptors (KAR) induces the activation of effector mechanisms by CL including expression and release of death ligands like FasL (Fas ligand) and TRAIL (TNF-related apoptosis inducing ligand) {Takeda, 2001 #2970; Screpanti, 2005 #2988; Anel, 1994 #2991} and the granule exocytosis {Bossi, 2005 #334; de Saint Basile, 2010 #2926} (Fig. 1). The granule exocytosis pathway is rapidly executed by a directional mobilization of preformed specialized cytoplasmic granules, toward the contact site of CL and target cell, an structure known as immunological synapse, where their content is released {Bossi, 2005 #334; de Saint Basile, 2010 #2926}. The pore forming protein, named perforin (PRF1) {Voskoboinik, 2006 #307} and a family of serine proteases, known as granzymes (granule associated enzymes, GZM) are the dominant constituents of the cytolytic granules {Chowdhury, 2008 #1205; Pardo, 2009 #385}. GZMA and GZMB have attracted most of the attention over the last decades. However additional GZMs with possible functional significance (in total 5 in human and 10 in mice) and other cytoplasmic granule-associated molecules like the human-specific protein granulysin {Krensky, 2009 #2927} have been described, though their biological

functions during cancer immunity and immunotherapy have not been elucidated {Pardo, 2009 #385; Bovenschen, 2010 #2908; Voskoboinik, 2015 #2958}.

In most cases PRF1 act as a vehicle for GZMs delivery into the cytosol of the target cell by a mechanism that seems to be dependent on its ability to form pores in membranes {Voskoboinik, 2006 #307}. Paradoxically, this event, one of the most critical steps controlling the elimination of cancer cells, is still a matter of intense debate and it 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 access to the target cell cytosol, although the nature of the pore is not clear {Lopez, 2013 #2930; Metkar, 2011 #2931}. However it is still unknown if the alternative models proposed (receptor- or clathrin-mediated GZM endocytosis and release from endosomes by co-endocytosed PRF1), operate under some circumstances depending on the target cell {Pipkin, 2007 #1067}. 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 lyses. This hypothesis is supported by experiments showing that rat basophil leukemia cells transiently transfected with *PRF1* cDNA lyse Jurkat cells {Voskoboinik, 2004 #2994}. On this context changes in the lipid composition of the plasma membrane in cancer cells may influence its response (either as GZM-delivery or as lytic agent) to PRF1, modulating the sensitivity of cells to CL and immunotherapy {Lehmann, 2000 #2932; Antia, 1992 #2933}.

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 {Andrade, 2010

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#2959} as well as extracellular matrix proteins controlling vascular integrity, inflammation and skin aging {Parkinson, 2014 #2996; Hiebert, 2012 #2997; Hendel, 2010 #2709} 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 physiological models.

Who is who during cell death induced by CL in cancer immunosurveillance and immunotherapy?

Cancer immunosurveillance

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 tumours as well as during chemical carcinogenesis (table 1 and {Bolitho, 2007 #333; van den Broek, 1996 #211; Pardo, 2002 #386; Davis, 2001 #36}). This also applies to control of cancer metastasis {Smyth, 1999 #2937; Bolitho, 2007 #333}. 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 {Kagi, 1994 #87}. The role of PRF1-mediated cell death in cancer immunosurveillance has been clearly established during spontaneous cancer development (table 1). This seems to be specially relevant for tumour of haematological origin since *PRF1* KO (knock out) mice develop spontaneous B lymphoma {Smyth, 2000 #325}. In addition, *PRF1* deficiency enhances the oncogenic potential of diverse proteins such Abl-1 (Abelson murine leukaemia viral oncogene homolog 1), Bcl-2 (B-cell lymphoma 2) or Mlh1 (MutL homolog 1)

{Bolitho, 2009 #2936}. Importantly, *PRF1* deficiency in humans seems to predispose to development of several types of lymphoma and leukaemia {Trapani, 2013 #2928}. Concerning other types of tumours it has been recently shown that *PRF1* deficiency accelerates the onset of Her-2/neu (human epidermal growth factor receptor)-driven breast carcinomas {Street, 2007 #2983; Macagno, 2014 #2982} as well as neoplastic grading {Macagno, 2014 #2982}.

Concerning the role of GZMs in cancer immunosurveillance the results are not so 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 {Revell, 2005 #324; Pardo, 2002 #386; Fehniger, 2007 #944}. In contrast others have reported that mice deficient in these *GZMs* control implanted tumours as well as chemical-induced sarcomas as efficient as wild type mice {Smyth, 2003 #185; Davis, 2001 #36}. Here it should be noted that in some models GzmB deficiency abrogates the function of CD4 T regulatory cells increasing the anti-tumour response of CL ({Cao, 2007 #338} and Own unpublished results) which may mask the anti-tumour potential of CL-associated GzmB.

Although these discrepancies have not been clarified yet it has been argued that other GZMs might compensate the absence of GZMA and GZMB, meanwhile PRF1 deficiency would inactivate the anti-tumour function of all GZMs. However, deficiency in other *GZMs* like *GZMM* {Pao, 2005 #467} 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 tumours is not increased since its cytotoxic potential *in vitro* is very low {Joeckel, 2011 #2853}. Here it may be

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possible that, in the absence of GZMs, PRF1 *per se* would eliminate cancer cells by inducing cell lysis in a similar way as the complement membrane attack complex as previously suggested {Voskoboinik, 2006 #307} (Fig. 2).

In some cases cell death induced by *PRF1* deficient CL can be restored at longer incubation times in specific tumour cell types that presents sensitivity to FasL , suggesting that this death ligand may also contribute to tumour immunosurveillance *in vivo* {Kagi, 1994 #91; Lowin, 1994 #232; Screpanti, 2005 #605}. In fact, it has been recently shown that Tc cells use FasL to eradicate transplanted B cell lymphoma cells in RAG1 (recombination activating gene 1) deficient mice {Afshar-Sterle, 2015 #2978}, confirming previous findings using animals with natural mutations in Fas (lpr) or FasL (gld) {Peng, 1996 #2979; Davidson, 1998 #2980}. Supporting these studies, the presence of Fas mutations in human lymphoproliferative disorders was correlated with a higher incidence of B and T lymphoma {Price, 2014 #2992; Straus, 2001 #2981}. However, in this case, development of lymphoma could be related to a defect in activation-induced cell death rather than FasL-mediated immunosurveillance. Of note, it has not yet been reported a similar incidence in human with FasL mutations.

Regarding the other major death ligand, TRAIL, it was firstly 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 the regulation of the immune response {Martinez-Lostao, 2013 #2993; Falschlehner, 2007 #2964}. The role of endogenous TRAIL in tumour immunosurveillance is not fully understood yet. A few studies have clearly shown that NK cells are able to kill cancer cells using TRAIL {Cretney, 2002 #2962; Takeda, 2001 #2970}. Indeed, it seems that TRAIL is a

mechanism used by liver but not spleen NK cells to prevent tumour metastasis. It has been shown that TRAIL or TRAIL-R (TRAIL-receptor) deficient mice are more susceptible to some transplanted tumours {Sedger, 2002 #2968} as well as chemical carcinogenesis {Cretney, 2002 #2962; Finnberg, 2008 #2965}. Animals deficient in TRAIL and TRAIL-R do not spontaneously develop tumours in early age {Cretney, 2002 #2962; Diehl, 2004 #2963}, but aged TRAIL deficient mice presents an slightly increased susceptibility to develop spontaneous lymphoma {Zerafa, 2005 #2977}. This susceptibility seems to be more pronounced in the context of loss of at least one allele of p53 {Takeda, 2001 #2970; Zerafa, 2005 #2977}. However other groups found that development of spontaneous tumours was not increased in TRAIL-R-deficient mice in the absence of p53 {Yue, 2005 #2976}.Despite these findings, its main role seems to be related to the control of tumour metastasis. This effect was originally shown using transplanted syngeneic cell lines {Cretney, 2002 #2962; Smyth, 2001 #2969; Takeda, 2002 #2971; Takeda, 2001 #2970} and later confirmed in a more physiological model of spontaneous metastasis after chemical-induced carcinomas {Grosse-Wilde, 2008 #2966}. In contrast to PRF1 and Fas, there have not been described any human mutations in TRAIL or TRAIL-R that correlate with a higher predisposition to cancer development.

Cancer Immunotherapy

In contrast to cancer immunosurveillance the involvement of PRF1 and GZMs during cancer immunotherapy has not been explored in deep and the available results are not clear. The potent immunodominant Tc cell epitope, lymphocytic choriomeningitis virus (LCMV) peptide gp33, has been widely used as a model tumour antigen to analyse 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 cells lines of diverse origin depends of the presence of PRF1 and is not affected by FasL deficiency (manuscript under preparation).

. Several works have pursued to reveal the role of PRF1 and Death Ligands in the elimination of cancer cells by immune modulators used in clinics like immuno-stimulatory antibodies or cytokines (Table 1). However the results are difficult to interpret since generally different tumour models have been employed. A summary of the results obtained using immune modulators is depicted in figure 1 and table 1.

As shown in Table 1 *in vivo* elimination of colon, prostate and breast carcinoma cell lines mediated by anti-CD73, anti-CTLA-4, anti-PD-1 monoclonal antibodies (mAb) or the combination of them is not affected by the absence of PRF1 {Morales-Kastresana, 2013 #2938}. In contrast, PRF1 but not by FasL or TRAIL was shown to contribute significantly to the anti-tumoral effect of the combination of BRAF (B-Raf proto-oncogene, serine/threonine kinase) inhibitors and agonistic anti-CD137 antibody in melanoma cells (table 1) {Knight, 2013 #2945}. We have recently found that anti-CD137 mediated elimination of EL4 lymphoma in mice depends on both PRF1 and FasL {Morales-Kastresana, 2013 #2938}. It will be required more experimental evidence to find out if the contribution of PRF1 in mAb-mediated control of tumours is dictated by the type of cancer cell or by the type of stimuli.

Regarding cytokine therapy most studies have been focused in cytokines that predominantly activate NK/NKT cell-mediated responses. The elimination of melanoma and sarcoma murine cell lines by IL-12 {Song, 2000 #2943; Sin, 2012 #2944} or IL-15 {Liu, 2012 #2947} was dependent on PRF1 expression. PRF1 was also required to control melanoma tumour metastasis by IL-12 {Schultz, 1999 #2939;

Kodama, 1999 #2940}. In contrast *PRF1* deficiency did not affect IL-12 and αGalCer (alpha-galactosylceramide)-mediated control of liver metastasis using the RENCA renal carcinoma model. In this case metastasis was inhibited by anti-TRAIL therapy {Smyth, 2001 #2969}. Another study confirmed that *PRF1* deficiency did not affect the anti-metastatic activity of αGalCer in the B16 melanoma model {Hayakawa, 2001 #2989}. Finally, it was shown that the anti-tumour effect of IL-12 against melanoma cells in mice was dependent on PRF1 {Hashimoto, 1999 #2941}. In contrast the same study showed that IL-18 activity was dependent on FasL and independent of PRF1. As previously suggested it seems that the effect of IL12 during cancer immunotherapy is model dependent {Smyth, 2000 #3001}.

Another approach used to treat cancer is the so called adoptive cell transfer (ACT) that consists of the administration to the cancer-
with direct anticancer activity. Some works have also analysed the effector molecules of CLs involved in cancer elimination during ACT. It was found that Tc or NK cells from *PRF1*-deficient mice control as efficiently as wt cells lung metastasis in the B16 melanoma model {Takeda, 2011 #3004; Dobrzanski, 2001 #3003}. In a recent study Phil Darcy´s group has shown that PRF1, GZMB and GZMM are required to inhibit the growth of a transplanted sarcoma cell line during adoptive NK cell transfer {Pegram, 2010 #2949}. In addition it was shown that TRAIL {Joeckel, 2014 #2950} was required for the anti-tumour function of transferred NK cells. In contrast, the absence of GZMA did not affect tumour growth. The lack of anti-tumoural activity of GZMA in this model maybe explained by the last findings questioning the cytotoxic potential of this and other GZMs {Metkar, 2008 #378; Kaiserman, 2006 #310; Joeckel, 2014 #2950}.

A conclusion that can be reached from these studies is that in some types of cancer the cell death executors involved in cancer immunosurveillance maybe different from those activated by immunotherapy. This hypothesis is strongly supported when comparing studies analysing immunosurveillance and immunotherapy in similar tumour models (table 1). i.e.: *GZMB* deficient mice are more susceptible than wild type mice to sarcomas induced by MCA {Davis, 2001 #36}, but they are compromised in the control of implanted sarcomas during adoptive NK cell transfer {Pegram, 2010 #2949}. *PRF1* deficient mice are more susceptible than wild type mice to oncogene-driven or to implanted mammary carcinomas {Smyth, 1999 #2937; Street, 2007 #2983; Macagno, 2014 #2982}, but they have no problem to control implanted mammary carcinoma cells during mAb therapy {Morales-Kastresana, 2013 #2938}. *PRF1* deficiency increases the susceptibility to liver and lung metastasis in the RENCA renal carcinoma model {Abdool, 2006 #3000} but it does not affect the control of metastasis during IL12/αGalCer {Smyth, 2001 #2969} or adoptive Tc cell therapy {Seki, 2002 #2998}. *PRF1* deficiency increases the susceptibility to implanted prostate cancer cell line RM1 {Smyth, 1999 #2937}, but this deficiency has not impact on mAb-mediated control of this cell line {Morales-Kastresana, 2013 #2938}.

The differences observed in the molecules involved in the elimination of cancer cells during immunosurveillance and/or immunotherapy could be related to the strength of the stimuli recognised by the CL as recently suggested {Shanker, 2009 #3005}

Who makes what during cell death induced by CL??

Apoptotic and non-apoptotic 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 (Fig. 2) {Voskoboinik, 2015 #2958}. However, this overreaching conclusion mainly obtained from *in vitro* models using purified GZMs delivered with a great variety of agents, may not be true. In contrast recent evidences indicate that neither all GZMs present cytotoxic potential nor the mechanism of cell death activated by CL is always apoptosis (Fig. 2) {Pardo, 2009 #385; Joeckel, 2014 #2950}.

First of all, it is worth mentioning again that PRF1 alone may be able to lyse specific target cells under the abovementioned circumstances. This effect has only been shown in Jurkat cells using as effector cells Rat Basophil Leukaemia cells transfected with *PRF1* cDNA and it should be confirmed employing CLs. This lytic activity would 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 Tc and NK cells from GZMA and GZMB double KO mice still present some ability to induce cell death on tumour target cells *ex vivo* {Simon, 1997 #177; Pardo, 2004 #387; Davis, 2001 #36} as well as during *in vivo* peritoneal killing {Hoves, 2011 #2954}, although at a reduced level in comparison with CL from wild-type mice. However this type of cell death does not present clear apoptotic features proceeding with membrane permeabilisation in the absence of caspase activation {Pardo, 2004 #387; Waterhouse, 2006 #304}. Notably, it was later shown that cells eliminated in the absence of GZMA and GZMB did not show eat-me signals for DC and did not induce antigen cross-presentation due to reduced phagocytosis of dead tumour cells {Hoves, 2011

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#2954}. Supporting this finding, we found that GZMB was required for immunogenic calreticulin exposure in plasma membrane of the dying cells during Tc cell attack {Pardo, 2009 #2955}.

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 {Bovenschen, 2010 #2908; Ewen, 2011 #2925; Chowdhury, 2008 #1205}. Excluding GZMB that clearly activates apoptosis involving both caspases {Fulda, 2015 #3006} as well as the mitochondrial intrinsic pathway regulated by the Bcl-2 family {Gibson, 2015 #3008; Ewen, 2011 #2925}, the molecular mechanism of cell death activated by purified GZMs is not apoptotic {Bovenschen, 2010 #2908; Ewen, 2011 #2925} and often contradictory (Fig. 2) {Hoves, 2010 #2902} and will not be the focus of this review (reviewed in {Ewen, 2011 #2925}). Moreover, it is not clear if these GZMs are indeed inducing cell death when delivered by CL. Indeed, CL from GZMM or GZMA KO mice does not present any defect to kill most target cells. Recent evidences from several independent groups combining data generated using both purified molecules as well as CL indicate that the cytotoxic potential of human GZMA is very low if any {Joeckel, 2014 #2950; Wensink, 2015

#1; Metkar, 2008 #378; Kaiserman, 2006 #310}. In mice it has been described that GZMA may induce cell death in specific cancer cell lines {Pardo, 2002 #142} by a mechanism that requires an intact actin cytoskeleton. This process does not resemble all features of apoptosis and has been named athetosis {Susanto, 2013 #2934}.

Several experimental evidences using intact CL have shown that GZMB induces apoptosis in target cells. CL from mice deficient in the *GZMB* cluster are unable to induce fast oligonucleosomal DNA fragmentation {Heusel, 1994 #2914} and PS translocation in the absence of membrane permeability {Pardo, 2008 #390}. Indeed GZMB has been shown to be crucial for CL-mediated caspase-3 and Bid activation {Pardo, 2008 #390} and degradation of specific intracellular substrates such as tubulin {Adrain, 2006 #285; Goping, 2006 #303}, Mcl-1 (myeloid cell leukaemia-1) or Bcl-X$_L$ (B-cell lymphoma-extra large) {Catalan, 2015 #2952} in tumour cells still presenting an intact plasma membrane (Fig. 2). Notably, the mechanism of cell death activated by GZMB maybe dependent on the species {Kaiserman, 2006 #310} as well as on the type of cell transformation {Catalan, 2015 #2952}. In humans it has been shown that cell cytotoxicity of cytokine-activated human NK cells in which GZMB is blocked, is greatly reduced {Sedelies, 2008 #1382; Mahrus, 2005 #526} confirming that GZMB is the main cell death inductor in CLs. To confirm that cell death induced by GZMB is important during Tc cell-mediated cancer immunotherapy we have employed the LCMV gp33 antigen model. Here we have found that Tc cells require GZMB-mediated cell death to prevent development of tumours at long term (Manuscript under preparation).

However, cell death induced by CL through GZMB is not always apoptotic in nature. This fact is particularly evident when target cells in which apoptotic pathways are blocked are employed. 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 {Pardo, 2008 #390}, highlighting the potential benefits of immunotherapy to treat cancer cells that do not respond to conventional therapy {Gibson, 2015 #3008; Fulda, 2015 #3006}. We have recently confirmed in humans that allogeneic activated NK

cells expressing GZMB eliminate haematological cancer cells in which apoptosis is blocked by p53 mutation, over-expression of Bcl-X_L even in the presence of caspase inhibitors {Sanchez-Martinez, 2015 #2953}. However, under these circumstances the phenotype of dying cells is not apoptotic and PS translocation did not preceded 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 CL in the absence of GZMB or in target cells in which apoptosis is blocked may not be enough to amplify the anti-tumour immune response and establish anti-tumour memory that prevents future tumour relapse.

Other modalities of cell death

As indicated above several experimental evidences indicate that granule exocytosis could induce cell death independent of apoptosis.. At present it is unknown if other mechanism of cell death and/or survival such pyroptosis, necroptosis {Fulda, 2015 #3006} or autophagy {White, 2015 #3007} 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 IL-1β, TNF-α and IL-6 by macrophages in a caspase-1 dependent manner (Fig. 2) {Metkar, 2008 #378; Anthony, 2010 #2841}. Indeed, *GZMA* deficient mice resist sepsis without compromising other protective functions like Tc- cell mediated elimination of infected macrophages {Arias, 2014 #2984}. However, caspase-1 activation does not lead to macrophage cell death in these conditions as in the case of pyroptosis induced by bacterial infection.

Death ligands

It is known from long time ago that death receptor ligation leads to caspase-dependent apoptotic cell death (Fig. 2) {Fulda, 2015 #3006}. However, the mechanism of cell death shifts from apoptosis to necroptosis in the presence of caspase inhibitors {Vandenabeele, 2010 #2986} (Fig. 2). The molecular mechanism of death receptor-induced necroptosis, that involves the kinases RIP1 (receptor-interacting protein 1) and RIP3, has been described recently {Vandenabeele, 2010 #2986}. The possibility that tumour cells resistant to death receptor-induced apoptosis could shift their way of cell death towards necroptosis could have an impact on immunogenicity and the subsequent action of immune surveillance mechanisms as well as on immunotherapy treatments.

On the other hand, TRAIL not only can induce apoptosis and/or necroptosis but also pro-inflammatory responses through the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), PKB/Akt (protein kinase B/RAC serine-threonine-protein kinase) and MAPK (mitogen-activated protein-kinase) pathways {Falschlehner, 2007 #2964} (Fig. 2). This could be exploited by tumour cells for their own benefit promoting proliferation, migration and invasion of cancer cells {Azijli, 2013 #2960; Trauzold, 2006 #2973}. Indeed, in a pancreatic adenocarcinoma xenograft model, it has been described that tumour cells used TRAIL to promote the development of metastases in liver {Trauzold, 2006 #2973}. In this line it was found that FasL also enhances motility and invasiveness in apoptosis resistance cancer cells {Barnhart, 2004 #2995}. More recently, it has been shown that signalling through TRAIL receptors can be used by tumour cells to promote KRAS

(Kristen rat sarcoma viral oncogene homolog)-driven tumorigenesis {von Karstedt, 2015 #2974}.

Concluding remarks

CL (Tc and NK cells) are the main effector cells executing transformed cells during cancer immunosurveillance and immunotherapy. However, the experimental evidences suggest that the molecular mechanisms involved in immunosurveillance are not always the same as those in immunotherapy. PRF1 and GZMB is the most potent pathway used by CL to kill cancer cells, overcoming anti-apoptotic mutations including p53 deletion/mutation, over-expression or down-regulation 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 *PRF1* deficiency. Originally the main effector pathways of CL, PRF1/GZMs and death ligands, were thought to act exclusively by inducing apoptotic cell death on transformed cells. Recent experimental evidences indicate that during the interaction between CL and tumour cells, non-apoptotic 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 on how CL actually kill cancer cells during immunotherapy will help to predict patient responses and to select the best protocols to get activated CL that efficiently kill tumour cells without inducing other undesirable effects.

References

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

| Mouse gentoype | IMMUNOSURVEILLANCE[1] | IMMUNOTHERAPY[2] | | | |
		cytokines	CTLA-4 PD-1/PD-L1	ACT	CD137
Prf^{-/-}	↑Spontaneous B cell lymphoma[Smyth, 2000 #325; Bolitho, 2009 #2936] ↑Oncogen (TP53, v-Abl, Bcl-2, Mlh-1) - driven B cell lymphoma[Bolitho, 2009 #2936] ↑MCA-induced sarcoma[Davis, 2001 #36] ↑HER2/Neu-driven mammary carcinoma[Street, 2007 #2983; Macagno, 2014 #2982]	< IL12[Sin, 2012 #2944] {Schultz, 1999 #2939; Kodama, 1999 #2940; Song, 2000 #2943} {Hashimoto, 1999 #2941} < IL15[Liu, 2012 #2947] = IL12[Smyth, 2001 #2969] = αGalCer[Smyth, 2001 #2969; Hayakawa, 2001 #2989]	= Prostate, colorectal and breast carcinoma[Allard, 2013 #2942]	< RKIK sarcoma[Pegram, 2010 #2949]	< EL4 lymphoma[Morales-Kastresana, 2013 #2938]
GzmA^{-/-}	= MCA-induced sarcoma[Davis, 2001 #36]			= RKIK sarcoma[Pegram, 2010 #2949]	
GzmB^{-/-}	= MCA-induced sarcoma[Davis, 2001 #36]			< RKIK sarcoma[Pegram, 2010 #2949]	
GzmAxB^{-/-}	= MCA-induced sarcoma[Davis, 2001 #36]				
GzmM^{-/-}				< RKIK sarcoma[Pegram, 2010 #2949]	
TRAIL^{-/-}	↑Spontaneous B lymphoma[Zerafa, 2005 #2977] ↑Oncogen (TP53) - driven B cell lymphoma[Zerafa, 2005 #2977] ↑MCA-induced sarcoma[Sedger, 2002 #2968; Finnberg, 2008 #2965; Cretney, 2002 #2962] = HER2/Neu-driven mammary carcinoma[Zerafa, 2005 #2977]	< αGalCer (antiTRAIL ab)[Smyth, 2001 #2969]		< RKIK sarcoma[Pegram, 2010 #2949]	
TRAIL-R^{-/-}	↑Oncogen (Eμ-Myc) - driven B cell lymphoma and associated lung metastasis[Finnberg, 2008 #2965] ↑DEN -induced hepatocarcinoma[Finnberg, 2008 #2965] ↑radiation-induced T lymphoma[Finnberg, 2008 #2965] = DMBA/TPA-induced primary squamous cell carcinoma[Grosse-Wilde,				

	2008 #2966}		
Lpr [3]	↑Metastasis during DMBA/TPA-induced squamous cell carcinoma[Grosse-Wilde, 2008 #2966] ↑Spontaneous B cell lymphoma[Peng, 1996 #2979] ↑Spontaneous plasmocytoid tumours[Davidson, 1998 #2980]		
FasL [-/-]		< B cell lymphoma[Afshar-Sterle, 2015 #2978]	
Gld [3]	↑Spontaneous plasmacytoma	= RKIK sarcoma[Pegram, 2010 #2949]	< EL4 lymphoma[Morales-Kastresana, 2013 #2938]

[1] Susceptibility of the corresponding mouse strain to chemical, spontaneous and oncogene-driven carcinogenesis.

[2] Efficacy of different immunotherapy protocols in the corresponding mouse strains; = no change in comparison with wild type mice; < less efficacy than in wild type mice.

[3] Gld and Lpr are mouse strains with natural mutations for FasL and Fas, respectively.

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Figure legends

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 tumour antigen-derived peptide bound to a MHC class I molecule as well as co-stimulatory signals (CD8 and CD28). Activation of NK cells (NK) relies on the balance between activating and inhibitory receptors provided by tumour cells. Although the mechanisms of activation of Tc and NK 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 centre (MTOC) rapidly polarizes the traffic of preformed secretory granules towards the presynaptic membrane. The secretory granules then fuse with the plasma membrane at the immunological synapse and release their content (perforin and granzymes) leading to tumour elimination. Deficiency in proteins controlling intracellular trafficking and granule fusion and release affects exocytosis at different levels reducing the ability of CL to kill target cells {de Saint Basile, 2010 #2926}. During death ligand/death receptor-mediated apoptosis, upon CL reactivation, preformed FasL (Fas ligand) and TRAIL (TNF-related apoptosis inducing ligand) are expressed on the surface of CL or released as exosome membrane-bound death ligands after fusion of multivesicular bodies (MVB) with the cell-cell contact zone. Reactivation of CL 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 CL are able to kill susceptible tumour cells through their interaction with their respective death receptors. Activation these effector mechanisms can be modulated by several mAb with immunomodulatory activity enhancing anti-tumour activity of CL. mAb can bind to Fc receptors expressed by NK cells allowing antibody-dependent cell cytotoxicity (ADCC). Blocking mAb against immune checkpoint molecules (CTLA-4 and PD-1) prevents, respectively, inhibitory or cell death signals that CL receive from these molecules. Finally, agonistic mAb against co-stimulatory molecules such as CD137 lead to the increase of CL cytotoxic activity against tumour cells.

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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 pro-apoptotic 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) oligomerisation on the mitochondrial outer membrane allowing the release of cytochrome C (cyt C) from mitochondria. Once in the cytoplasm, cyt C, apoptotic protease activating factor 1 (Apaf-1), and procaspase-9 form a multimolecular complex named 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 pro-apoptotic Bcl-2 family protein Bim from its association with anti-apoptotic proteins Mcl-1 (myeloid cell leukaemia-1) and Bcl-X_L (B-cell lymphoma-extra large).

Granzyme A regulates the production of pro-inflammatory cytokines (IL-1β) by a mechanism dependent on caspase-1. The contribution of the inflammasome platforms to this process is suggested although not proven yet. In *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 ($\downarrow \Delta\Psi M$) and reactive oxygen species (ROS) production. ROS generation in turn induce DNA damage and the subsequent activation of DNA-repairing mechanisms, among them, the SET complex which translocates from the ER (endoplasmic reticulum) 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 physiological relevance of this ability is questioned {Pardo, 2009 #385; Joeckel, 2014 #2950; Voskoboinik, 2015 #2958}. In addition, it has also been

reported that granzymes K and M can also regulate the production of pro-inflammatory cytokines.

Induction of cell death of tumour cells by CL induces phosphatidylserine translocation of calreticulin and maybe other danger signals like ATP (adenosine triphosphate). These events are necessary for a proper activation of the immune system against the dying tumour 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 oligomerisation. Consequently, the oligomerised 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 trans-activation. 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 pro-apoptotic protein Bid, generating tBid, which, as described above, activate 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 (RIP)1 and RIP3, forming a complex named necrosome which phosphorylates MLKL (mixed lineage kinase domain-like protein) promoting its oligomerisation. Then, MLKL inserts into and permeabilises 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-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and of mitogen-activated protein kinases (MAPK) and Akt kinase (protein kinase B), promoting survival signals.