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REVIEW ARTICLE

The intrinsic immunogenic properties of cancer cell lines, immunogenic cell death, and how these influence host antitumor immune responses

Tania Løve Aaes[1,2,3] · Peter Vandenabeele[2,3,4]

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Abstract

Modern cancer therapies often involve the combination of tumor-directed cytotoxic strategies and generation of a host antitumor immune response. The latter is unleashed by immunotherapies that activate the immune system generating a more immunostimulatory tumor microenvironment and a stronger tumor antigen-specific immune response. Studying the interaction between antitumor cytotoxic therapies, dying cancer cells, and the innate and adaptive immune system requires appropriate experimental tumor models in mice. In this review, we discuss the immunostimulatory and immunosuppressive properties of cancer cell lines commonly used in immunogenic cell death (ICD) studies being apoptosis or necroptosis. We will especially focus on the antigenic component of immunogenicity. While in several cancer cell lines the epitopes of endogenously expressed tumor antigens are known, these intrinsic epitopes are rarely determined in experimental apoptotic or necroptotic ICD settings. Instead by far the most ICD research studies investigate the antigenic response against exogenously expressed model antigens such as ovalbumin or retroviral epitopes (e.g., AH1). In this review, we will argue that the immune response against endogenous tumor antigens and the immunopeptidome profile of cancer cell lines affect the eventual biological readouts in the typical prophylactic tumor vaccination type of experiments used in ICD research, and we will propose additional methods involving immunopeptidome profiling, major histocompatibility complex molecule expression, and identification of tumor-infiltrating immune cells to document intrinsic immunogenicity following different cell death modalities.

Facts

- During immunogenic cell death (ICD) cancer cells are classically undergoing apoptosis, but also necroptosis has been studied as another regulated form of cell death, which triggers an adaptive immune response.
- Most cancer cell lines used in ICD studies express endogenous, often retrovirally derived, antigens as well as various degrees of immunostimulatory and immunosuppressive molecules.
- The efficacy of immunotherapy relies on antigenic epitope recognition and killing of cancer cells.

Open questions

- What are the consequences of using cell lines with high expression of endogenous tumor antigen in experimental immunity studies?
- Are some cell death modalities superior to others in terms of inducing ICD?
- Do different cell death modalities influence the intrinsic immunogenic properties of cancer cell lines?
- Should the experimental tumor vaccination strategies in mouse studies be revisited?

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✉ Peter Vandenabeele
peter.Vandenabeele@irc.vib-urgent.be

[1] Unit for Cell Clearance in Health and Disease, VIB Center for Inflammation Research, Ghent, Belgium

[2] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

[3] Cancer Research Institute Ghent (CRIG), Ghent, Belgium

[4] Unit of Molecular Signaling and Cell Death, VIB Center for Inflammation Research, Ghent, Belgium

Immunogenic cell death (ICD) and tumor vaccination studies

Tumor antigenicity and vaccinations

To obtain an efficient cancer vaccine, it was recently proposed that the diligent choice of four main elements was required: tumor antigens (tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs)), formulations (e.g., cell based, antibody based, and vector based), immune adjuvants (e.g., cell death derived factors, TLR agonists, and GM-CSF), and delivery vehicles (e.g., emulsion and liposomes) [1]. For a successful antitumor immune response to be achieved, high levels of tumor antigen expression are needed to reach the threshold for T-cell recognition, thereby breaking immunological tolerance [2–4]. Tumor antigens can be of different origin and specificity, and while various definitions have been used in the past, we would make the main distinction between TAA and TSA (Table 1).

TAAs include genes that are found overexpressed in cancer tissues but not in normal tissues. In human cancers such overexpressed antigens include HER2/ERBB2, TERT, and BIRC5. As an example, HER2 was recently targeted by an antibody-drug (i.e., anthracycline) conjugate, which induced an immunogenic form of cell death in breast cancer cells, protected mice against a tumor challenge, and enhanced tumor eradication when combined with a checkpoint inhibitor [5]. TAAs that are involved in tissue differentiation tend to originate from a specific cell lineage such as mammaglobin-A and -B in the mammary gland, prostate-specific antigen, and the melanocyte-deriving antigens MART1 and PMEL. Common to these TAAs are thus that they are found expressed preferentially by cancer cells but not in normal tissues [6]. A third type of TAA is cancer-testis antigens (CTAs), which are a specialized subset of TAAs thought to provide higher tumor specificity, since they are not expressed in normal adult tissues. They include more than 60 genes whereof some of the most studied antigens are MAGE, SAGE1, and NY-ESO-1 [1, 7]. Finally, there is the class of oncofetal cancer antigens, which are expressed during fetal development but only limitedly in adult tissues [1]. It is obvious, that the immune response to the TAAs described above is controlled by central tolerance since they are not unique for the tumor. So, the immunotherapeutic challenge in case of TAAs is to break this tolerance.

TSAs can be of oncogenic viral origin and are thus specific for virus-induced cancers like those of human papilloma virus. Viral antigens are expressed only by the cancer, and are therefore seen as foreign antigen material to the host immune system, making them ideal for usage in anticancer vaccines [8]. Highly specific to tumor tissues only are also tumor neoantigens (TNAs), which arise as somatic mutations and are thus not only tumor specific but also highly immunogenic as they lack central tolerance [9]. With the recent advances in next generation sequencing and prediction of major histocompatibility complex (MHC)-binding epitopes, TNAs can be identified on an individual patient basis and used in precision medicine [10]. Several studies have shown that a higher neoantigen load is positively associated with higher T-lymphocyte cytotoxic activity and better prognosis [11, 12]. And last but not the least, tumor-specific neoantigens are bona fide tumor rejection antigens and hence highly suitable for cancer vaccinations [13].

Two independent mouse studies were the first to show that tumor-specific neoantigens can be identified using a tumor exome-based approach in which all mutations that resulted in novel possible MHC-binding peptides were predicted, and these potential neoantigens were, in turn, used to test cytotoxic T lymphocyte (CTL) antitumor reactivity and cytokine production (i.e., interferon gamma, IFN-γ) [14, 15]. Also in human cancers the mutational load and hence the prevalence of neoantigen formation can vary a lot depending on the cause and type of tumors [16]. Melanoma has the highest frequency of neoantigens with more than ten somatic mutations per megabase, yet among these neoantigens not all are efficiently recognized by autologous T lymphocytes [17]. Besides melanoma also bladder and lung carcinoma (e.g., non-small-cell lung carcinoma, NSCLC) are characterized by high mutation load [18, 19].

In mice, cancer immunology has been studied mainly by transplantable tumor models using well-characterized cancer cell lines such as: B16 melanoma, CT26 colon carcinoma, 4T1 breast carcinoma, EL4 T lymphoma, and Lewis lung carcinoma [20]. In such models, overexpression of model antigens (MAs)—ovalbumin (OVA) being the most commonly used—or retroviral gene products in cancer cell lines allow the further analysis of adaptive antitumor immunity and tumor antigen-specific cytotoxic T-cell responses [21].

Apoptosis and dead cell-derived adjuvants are keys to immunogenicity

Apoptosis is a programmed form of cell death, which occurs throughout the human body at a rate of one million events per second or hundreds of billion times a day during normal homeostasis [22]. Classically, apoptotic cells are phagocytosed even before signs of membrane permeabilization and internucleosomal DNA cleavage [23], and degraded by innate immune cells during a process, called efferocytosis, which is regarded as immunologically silent and that causes a tolerogenic immune response in the host [24]. However, under diseased conditions such as in cancer and during

Table 1 Tumor antigen classification and usage in vaccination studies.

Panel a: tumor-associated antigens (TAA)

Type of antigen	TAA example(s)	Associated cancer	Reference(s)
Overexpressed	HER2/ERBB2/Neu	Bladder, breast, cervical, colon, glioblastoma, lung, myeloma, esophageal, ovarian, pancreas, and prostate.	[143]
	TERT		[144]
	BIRC5/survivin		[145]
	AH1/Gp70 (*mu*)		[97]
Tissue differentiation	Mammaglobin-A and -B	B-cell lymphoma, melanoma, and prostate.	[146, 147]
	Prostate-specific antigen (PSA)		[148]
	MART1		[149]
	PMEL		[117]
Cancer-testis antigens (CTAs)	MAGE family	Bladder, breast, lung, melanoma, myeloma, and ovarian.	[150]
	SAGE1		[151]
	NY-ESO-1		[152]

Panel b: tumor-specific antigens (TSA)

Type of antigen	TSA example(s)	Associated cancer	Reference(s)
Oncogenic viral	HPV16 E6 and E7	Cervical, anal, oral, and vaginal.	[153]
Tumor neoantigens (TNAs)	*Unique to each tumor/ patient*	All tumors.	[14, 15, 154]
Model antigens (MAs)	Ovalbumin (OVA)	All tumors.	[155]

Grouping of the two major types of antigen with each of their subtypes and examples of antigens within each category. For each subtype of antigen, the types of cancer in which the respective antigens are used are listed. The references relate to the (first) antigenic epitope(s) associated with the respective antigen.

cancer therapy, the phagocytic capacity is insufficient and apoptotic cells expose and release molecules such as damage-associated molecular patterns (DAMPs), chemokines, and cytokines that are able to trigger an immune response—hence referred to as immunogenic apoptosis [25]. The first report on immunogenic apoptosis was described in relation to cancer therapy and was mainly based on studies that used anthracycline drugs as chemotherapeutics to kill cancer cells [26]. More specifically, the anthracycline drug doxorubicin was shown to induce an immunogenic form of caspase-dependent apoptosis in two different murine cancer cell lines: CT26 colon carcinoma and B16 melanoma cells. In the same study, two other related chemotherapeutic drugs, daunorubicin and idarubicin, were also shown to induce ICD. Since then, several other studies have followed, and ICD, especially in the form of apoptosis, has been described for other chemotherapeutic treatments like the anthracycline drugs mitoxantrone [27, 28], and oxaliplatin [29], photodynamic therapy using photosensitizers [30, 31], and radiation therapy [32].

A main characteristic of ICD and something that distinguishes it from the classic tolerogenic form of apoptosis is the increased exposure of endogenous adjuvants—otherwise intracellular molecules, DAMPs—to the extracellular compartment thereby triggering an immune response [33, 34]. DAMPs can be actively secreted, and act as "find-me-signals" that recruit dendritic cells to the dying tumor cells, examples of such are ATP [35–38] and high-mobility group box 1 protein (HMGB1) [39]. Other DAMPs are passively released, e.g., heat shock proteins (HSPs) HSP70 and HSP90 [40, 41], or exposed on the dying cell surface, e.g. calreticulin [27], and serve as "eat-me-signals" to the phagocyte. These and other DAMPs are described in more detail in other reviews [42–44]. Based on the vast amount of ICD literature that has emerged over the past decade, it is clear that ICD and the consecutive activation of the host immune system could be highly relevant for cancer therapy. Indeed, the engagement of the immune system results in a dual tumor eradication process: the primary cancer killing treatment, which is applied by the chemotherapy or radiotherapy, and second, an antitumor cytotoxicity response mediated by effector lymphocytes such as CTLs and NK cells by their secretion of cytotoxic molecules involving perforins, granzymes, and engagement of death receptors by surface expression of ligands such as Fas ligand and/or TRAIL [45, 46] (Fig. 1).

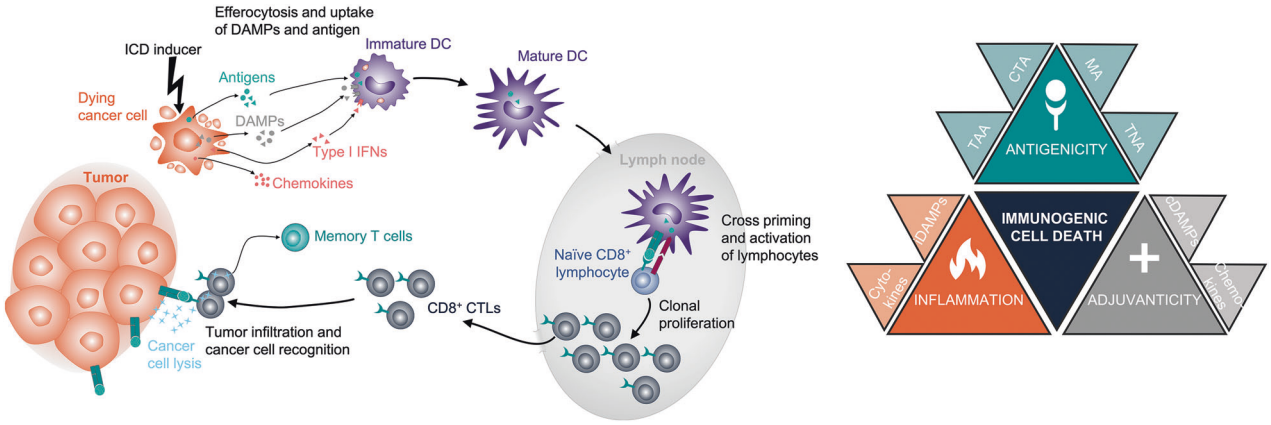

Fig. 1 The main elements and forces of an ICD tumor response. During cancer cell death by an ICD inducer (left panel), the release of DAMPs, chemokines and, cytokines, combined with the processing and presentation of tumor antigens on the cancer cell surface, will attract antigen-presenting cells like DCs. Following efferocytosis, the DCs migrate to the lymph nodes, where they cross-present the processed tumor antigens to NK and T lymphocytes. Combined with the effect of co-stimulatory factors such as CD80 and CD86, the lymphocytes become primed and activated in a process of cross-presentation. In turn, the now active CTLs and NK cells proliferate and travel to the tumor site, where they will recognize the tumor cells by expression of tumor antigenic epitopes, and induce their killing by secreting cytotoxic molecules involving perforins and granzymes and expressing death domain ligands such as FasL and TRAIL. The triforce of immunogenic cell death (right panel) consists of three main elements: inflammation, antigenicity (TAA, TNA, CTA, and MA), and adjuvanticity, with examples of each of these elements indicated in the small triangles on the edges.

Interaction between dying cancer cells and the immune system

Besides the classical uptake through phagocytosis and processing pathway involving phagosome fusion with the endoplasmic reticulum (ER), degradation by the proteasome, and translocation to the ER lumen by transporters associated with antigen presentation (TAP) before being loaded onto MHC class I molecules [47], tumor antigens can also be delivered to DCs by microvesicles released from the cancer cell [48]. Tumor cell-derived microparticles from UV-irradiated CT26 and B16 tumor cells were shown to contain both tumor antigens and endogenous DNA, and following phagocytosis by DCs, they resulted in a reduction in tumor growth in a vaccination model in BALB/c and C57BL/6 mice, respectively, in vivo [49]. In a direct MA model, murine fibrosarcoma cells that express vesicle membrane-bound MA—achieved experimentally by fusing the OVA peptide sequence to the C1C2 domains of lactadherin thereby allowing OVA to bind the vesicular, exosome, membrane [50]—were shown to grow slower than tumor cells that secreted soluble MA in immunocompetent mice, due to a more potent antigen-specific antitumor immune response against the exosome-bound MA [51]. Also in humans, DCs were demonstrated to engulf and process MA (i.e., ErbB2), which was carried in microvesicles from cancer cells, and cross-presented to CTLs, which, in turn, secreted IFN-γ [52]. The advances and current knowledge on exosome-derived tumor antigens and their effect on DCs in antitumor immunity have recently been summarized in a review [53].

In a dying tumor setting, DCs respond not only to antigens, but also to factors released from the tumor. Upon ICD induction in a tumor, DAMPs released together with chemokines (e.g., CXCL1, CXCL2, CCL2, and CXCL10) will attract phagocytes and antigen-presenting cells (APC) such as $CD103^+$ conventional dendritic cells (cDCs) to the tumor site [25, 54]. ICD can also be associated with the release of inflammatory cytokines such as interleukin-6 (IL-6), which promotes MHC class I expression on cDCs [55]. Following engulfment of the dying cell, also referred to as efferocytosis [56], the cDCs will, in turn, undergo maturation and transport tumor antigens back to the lymph nodes, where they cross-prime and activate CTLs [57, 58]. Thus, the combination of especially three main forces define the efficiency of an ICD response: (1) inflammation mediated by secreted factors such as cytokines (i.e., IL-6) and HMGB1, (2) antigenicity, which can be either tumor associated or of tumor-specific origin, and finally (3) adjuvanticity achieved by the secretion of various chemokines (such as CXCL1, CCL2, and CXCL10), type I interferons, and constitutive DAMPs (cDAMPs) [59–61] (Fig. 1). The recent reclassification of DAMPs is based on the nature and processing of these danger molecules. Within this terminology, cDAMPs are constitutively expressed endogenous molecules that are released upon cell death, e.g., ATP and HMGB1, while iDAMPs are generated or modified during the cell death process, e.g., cytokines and chemokines [60, 62].

Classically, the efficacy of an ICD response is determined by a combination of the following parameters: the efficiency of the dendritic cells to phagocytize the killed cancer cells in vitro, the strength of tumor antigen-specific killing of target

cancer cells by cytotoxic T cells ex vivo, or the level of antitumor protection obtained in vivo by prophylactic vaccination of mice with killed cancer cells followed by challenge with live cancer cells. The latter model represents the so-called golden standard method to test the ability of drugs to induce ICD [61, 63]. In this prophylactic tumor vaccination model, syngeneic mice are injected subcutaneously (s.c.) on one flank side with cancer cells killed in vitro by, e.g., a chemotherapeutic drug. One week later, the same mice are challenged with a live cancer cell injection s.c. on the opposite flank. An optimal ICD trigger will result in absence of tumor growth on the vaccination site due to efficient killing of the cancer cells in vitro, and also no tumor growth on the challenge site as a result of potent adaptive immune activation in the host by the vaccine [63, 64]. In order to establish an overview of the types of experiments that are used in vivo and ex vivo to detect ICD, we have summarized these methods and provided examples from the literature in which these methods were used to study anticancer responses (Table 2).

Necroptosis: an alternative and superior ICD mechanism?

ICD research was until a few years ago more or less restricted to cell death models involving apoptosis. Like in many human cancers, most murine cancer cell lines, which still represent the main research tool in ICD research, have a suppressed expression level of one of the crucial necroptosis executioner proteins, namely receptor-interacting serine/threonine-protein kinase 3 (RIPK3), while others lack the pseudokinase mixed lineage kinase domain like (MLKL), the target of RIPK3 [65]. Yet immunogenic studies show that the necroptotic key players, RIPK3 and MLKL, substantially contribute to immunogenicity of dying cancer cells (Table 3), which may explain their silencing in many human cancers [66, 67]. We and others have shown in several independent studies that necroptosis also can be considered an immunogenic form of cell death [68–72]. As in the case of immunogenic apoptosis, the necroptosis induction in several of these publications was shown to be associated with the release of DAMPs in the form of ATP and HMGB1. However, dependent on the cancer cell line studied, the stimulus given and whether dead-cell corpses or living cells that received a cell death stimulus were used, immunogenic necroptosis in some cases was also accompanied by NF-κB activation and continued cytokine release in vivo [62, 68], making it difficult to distinguish whether the cell death process as such or the combination with induced cytokines was implicated in the eventual prophylactic vaccination effect. Despite this and although the mode of necroptosis induction differed from RIPK3 oligomerization [68, 73], RIPK3 overexpression [69, 72], MLKL-encoding messenger RNA (mRNA) electroporation [71], to a combined cell death stimulus consisting of TNF, a synthetic second mitochondria-derived activator of caspases mimetic and z-VAD-fmk (together referred to as TSZ) [70], all these studies had a common denominator revealing an efficient adaptive immune response or protection against a challenge of live cancer cells in a prophylactic vaccination model in mice using the necroptotic cells as immunizers.

Another regulated cell death modality is pyroptosis—an inflammatory form of cell death—that can be triggered in cancer cells in response to chemotherapy [74], and is marked by a switch from an otherwise apoptotic phenotype to pyroptosis characterized by the cleavage of Gasdermin E (GSDME) and the consequent forming of pores in the plasma membrane [75]. GSDME-expressing cancer cells such as 4T1, B16, and CT26 are more prone to phagocytosis by tumor-infiltrating macrophages and increase the recruitment of NK cells and CTLs into the tumor bed [76].

With the definition of an increasing number of cell death modalities, it has become clear that ICD is not itself a particular mode of regulated cell death, but rather represents a multiparameter interaction modus between a dying cell and the immune system [59]. The key triggers of an ICD response have recently been redefined to not only consist of DAMPs (also known as cDAMPs) [60], but to include a cooperation of three elements: antigenicity (TAs), adjuvanticity (cDAMPs), and inflammation (inducible DAMPs (iDAMPs) such as cytokines and chemokines) [59]. Importantly, while an APC needs to encounter all of these three elements simultaneously in order to induce an ICD response, the three elements need not to derive from the dying cell alone, but can be produced by different cells in the tumor microenvironment. In this regard, a recurrent challenge in cancer therapy is the transformation of a "cold" poorly immune cell-infiltrated tumor (typically exemplified by prostate cancer) to a "hot" tumor, which is rich in tumor-infiltrating immune cells (e.g., melanoma and NSCLC) [77, 78]. For an efficient anticancer immune response to be induced by ICD, not only a "hot" TA-rich tumor microenvironment is thus needed, but also the presence of abundant adjuvants and inflammatory factors. Throughout the rest of this review, we will focus mainly on one of these three elements, namely antigenicity.

Endogenous tumor antigens and the impact on immunogenicity

Exploring neoantigen expression and the immunome of murine cancer cells

CT26 is one of the most used cell lines in the field of ICD and was generated in a laboratory in the 1970s by repeated intrarectal injections of the alkylating agent *N*-nitroso-*N*-methylurethane into BALB/c mice for a duration of

Table 2 Methods used to study ICD and tumor antigen responses in vivo and ex vivo.

Experiment	Description	Positive readout	References
In vivo ICD detection models			
Prophylactic tumor vaccination	Immunization s.c. with dying/killed cancer cells followed by challenge s.c. on the opposite flank with live cancer cells.	Antitumor protection on challenge site.	[29, 30, 63, 69, 71, 72, 129]
Tumor growth assay	Tumor growth after cell death induction in an established tumor in immunocompetent and/or immunodeficient mice.	Reduced tumor growth.	[26, 27, 71, 156]
Antigen-specific cytotoxic killing	Immunization s.c. with killed cancer cells, followed by intravenous injection of CFSE-labeled splenocytes that have been preincubated with the tumor antigen.	CFSE-labeled antigen-specific immune cells by flow cytometry.	[58, 71, 157]
Ex vivo ICD detection models			
Tetramer staining	Isolation of spleens/draining lymph nodes/peripheral blood from immunized mice or tumors after cell death induction.	Detection of $CD8^+$ $Tetramer^+$ CTLs by flow cytometry.	[26, 68, 69, 73, 156]
Antigen-specific IFN-γ release	IFN-γ release in response to antigenic epitope restimulation of lymphocytes isolated from immunized mice.	Increased IFN-γ signal by ELISpot assay or flow cytometry.	[28, 39, 69, 71, 72]
Immunophenotyping	Expression of cytotoxic molecules and checkpoint inhibitors on tumor cells.	Molecule expression detected by flow cytometry or IHC.	[158]
Lymphocyte cytotoxicity and degranulation	Granzyme B secretion by cytotoxic T cells and degranulation of NK cells as measured by CD107a surface expression.	Increased Granzyme B or CD107a expression by flow cytometry or IHC.	[159]
Lymphocyte proliferation assay	BrdU/EdU-supplemented drinking water of immunized mice, followed by isolation of splenocytes/lymph nodes.	$BrdU/EdU^+$ proliferating lymphocytes detected by flow cytometry.	[69]
Mixed lymphocyte reaction assay	Co-culture of T lymphocytes with tumor lysate-loaded DCs followed by BrdU labeling.	$BrdU^+$ proliferating lymphocytes detected by ELISA.	[160]
Chromium-release assay	Antigen-specific killing of ^{51}Cr-labeled cancer cells in co-culture with tumor antigen preincubated splenocytes.	^{51}Cr radioactivity measured in conditioned medium.	[26, 101]
Phagocytosis	Tumor-associated macrophage/DC phagocytic function.	Increased uptake of, e.g., fluorescently labeled tumor cells.	[27]

A summary of different experimental methods that have been used to demonstrate ICD in cancer studies. All of these methods share the common feature of tumor antigen dependency. The antigenic component derives either directly from the cancer cell line itself, or from synthesized, e.g., model antigens, antigenic epitopes, and tetramers.

6 months [79]. Colon tumor number 26 was the founding tumor of what was later to become the colon carcinoma cell line CT26. This clone was chosen due to its undifferentiated properties, its ability to reestablish a tumor when injected intraperitoneally and intravenously and because of its responsiveness to chemotherapies similar to the ones used to treat human colon cancer such as cyclophosphamide and 5-fluorouracil [80]. To date, ICD in murine cancer models has mainly been studied using the inbred mouse strains BALB/c, and its colon carcinoma-derived cell line CT26, or the C57BL/6 mouse-derived B16 melanoma and MCA-205 fibrosarcoma cell lines. While the B16 cell line originates from a spontaneous tumor [81], MCA-205 cells were also chemically induced by 3-methylcholanthrene-injection in C57BL/6 mice and generated by serial subcutaneous transplantation and single-cell derivation [82].

In cancer immunotherapy, the expression and cross-priming of tumor antigens are used as parameters for immune system activation and immunogenicity of a specific treatment or stimulus applied to the cancer cell. This is made possible due to the fact that tumor antigens are presented on the cancer cell surface by MHC molecules [83, 84]—in humans these are encoded by human leukocyte antigen proteins and depending on the genotype, this can also influence patient response to immunotherapy [85]. Antigen loading and presentation by MHC molecules have been extensively covered elsewhere [86], but in brief, antigenic peptides from intracellular origin are loaded on to MHC class I molecules and recognized by the T-cell receptor (TCR) on CTL, while when of exogenous origin, antigenic peptides are presented by MHC class II molecules typically expressed

Table 3 Necroptosis models in ICD and cancer immunogenicity.

Gene target	Cell line	Antigen	ICD readout	References
FADD (DD)	B16 (mu)	OVA (MA)	Antigen cross-presentation.	[161]
RIPK3	NIH-3T3 (mu)	OVA (MA)	Antigen cross-presentation, CTL cross-priming, tumor reduction.	[68, 162]
RIPK3	C4-I (hu)	–	DC activation.	[163]
RIPK3+/− FADD/DD	CT26 (mu)	AH1 (TAA)	Antigen cross-presentation, prophylactic tumor vaccination.	[69, 72]
RIPK3, MLKL	TC-1, El4 (mu)	–	Prophylactic tumor vaccination, therapeutic tumor growth model.	[70]
RIPK3	*NIH-3T3 (mu)	OVA (MA)	DC activation, CTL cross-priming.	[73]
MLKL	B16, CT26 (mu)	OVA (MA)	Therapeutic tumor growth model, prophylactic tumor vaccination, metastatic lung colonization, antigen cross-presentation.	[71]
RIPK3	*NIH-3T3 (mu)	mOVA (MA)	Cross-priming.	[62]
RIPK3	B16.F10, LL/2, *NIH-3T3 (mu)	OVA (MA)	Tumor reduction, antigen cross-presentation.	[164]

Reported roles of necroptosis key players RIPK3 and MLKL in cancer and/or immunogenic cell death models. Gene of interest (RIPK3 or MLKL), the cell line—mostly cancer cell lines—in question, the type of antigen and the immunogenic readout studied in the reported models.

*Non-cancerous mouse embryonic fibroblast cell line, *hu* human, *mu* murine, *MA* model antigen, *TAA* tumor-associated antigen, *mOVA* membrane-bound ovalbumin.

on professional APCs and recognized by the TCR on $CD4^+$ helper T cells. Importantly, APCs such as dendritic cells are also able to cross-present peptides of exogenous origin on MHC class I molecules [87].

In an elaborate CT26 study, it was shown that this cancer cell line expresses no MHC class II molecules but does express MHC class I molecules, subtypes H-2D, H-2K, and H-2L, at an extend between those of immune tissues (lymph node and spleen) and normal tissues [88]. Tumor cells expressing only MHC class I molecules were previously found to induce not only an antitumor CTL response, but also a helper T-cell immune response through the cross-presentation of antigens via APCs, which, in turn, will increase the antitumor immune response [89]. On the contrary, CT26 cells having a functional MHC class I but no MHC class II antigen-presenting mechanism is an interesting finding from a cancer therapy perspective, because some cancers develop mutations or defects in the MHC class I pathway and are thereby escaping CTL effector mechanisms [90]. However, in the CT26 cell line the MHC class I antigen-processing pathway appears intact and these cells can therefore present endogenous TAA and TSA-derived peptides from the cytosol directly for recognition by CTLs.

Despite the lack of MHC class II molecule expression by the CT26 cancer cell line, possible MHC class II-restricted neoantigenic epitopes have been characterized in CT26 and two other murine cancer cell lines, 4T1 mammary carcinoma and B16F10 melanoma [91], and immunization of naive mice with mRNA encoding these neoepitopes resulted in a substantial reduction in CT26 lung colonization [71, 91], confirming the important role of also CD4+ T lymphocytes in anticancer immunotherapy [92]. Not only does CT26 cells provide an immunogenic tumor microenvironment, but other indications reveal immunosuppressive characteristics such as, albeit low, surface expression levels of CD80 that bind with higher affinity to CTLA-4 than to CD28 [93], and that regulatory T cells infiltrate CT26 tumors in vivo, where they secrete IL-10 and transforming growth factor (TGF-β), both of which have suppressive effects on cytotoxic T-cell responses [94].

MuLV-derived tumor antigens define antitumor immune responses in mice

In the CT26 cell study by Castle et al., the striking finding was made that the most expressed gene in the CT26 transcriptome is gp70 [88]—a glycoprotein of 70 kDa which is one of two envelope (*env*)-encoded gene products deriving from a murine gamma leukemia retrovirus (MuLV). The MuLV *env* gene encodes a precursor protein, pr82, which is proteolytically cleaved into two mature proteins: gp70 (70 kDa) and p15E (15 kDa) [95, 96]. The gp70 gene is expressed in CT26 cells with more than 7000 reads per kilobase of transcript per million mapped reads (RPKM) in contrast to an average of 20 RPKM for other known cancer testes antigens [88]. Interestingly, CTLs recognize an immunodominant epitope of gp70 corresponding to $gp70_{423–431}$/H-$2L^d$, which is translated into what is known as the AH1 nonapeptide sequence: SPSYVYHQF [97]. Other murine cell lines also express the retroviral gp70 mRNA and give rise to tumor gp70-specific T-cell responses; such cell lines include the BALB/c-syngeneic 4T1 mammary carcinoma cell line and the C57BL/6-derived EL4 lymphoma, and B16 melanoma cells [98]. On the other hand in MCA-205 fibrosarcoma and MC38 adenocarcinoma cell

lines the p15E *env*-derived protein is expressed and its derived epitope $p15E_{604–611}$ (KSPWFTTL) is presented on MHC class I H-$2K^b$ molecules [99, 100].

Furthermore, gp70 is expressed not only in the murine cancer cell lines, but also in mice strains themselves. In BALB/c mice, the MuLV integration site is situated on chromosome 5, and in C57BL/6 mice it is found on chromosome 8 [101]. BALB/c mice, especially those of more than 8 months of age, show increased expression levels of gp70 transcripts. Consequently, gp70-deficient BALB/c mice develop a more diverse tumor antigen-specific CTL repertoire than the gp70-proficient mice [101]. Furthermore, CT26 tumor growth in gp70-deficient BALB/c mice is significantly increased upon depletion of the CTL compartment, hence confirming the importance of immune surveillance and anti-gp70/AH1 T-cell immunity in the control of CT26 tumor growth [101].

The immunogenic properties of gp70 and the immunodominant role of AH1

Since the original papers described the expression of MuLV DNA in different mouse strains [102] and the expression of MuLV-derived gene products in murine (cancer) cell lines [103], the first evidence of antigenic properties of gp70 was demonstrated already over 40 years ago [104, 105]. However, it would take another 15 years before the MuLV-derived gp70 gene product was thoroughly analyzed and the antigenic epitope of gp70 identified as the AH1 peptide sequence [97] (Fig. 2).

Since these initial publications, a vast number of studies have emerged over the past 20 years and have investigated the immunogenic properties of CT26 cells, the role of gp70 in antitumor immunity as well as the adaptive immune responses against the CT26-encoded AH1 epitope. First of all, immunization with recombinant gp70 apparently protects mice against a challenge with live CT26 tumor cells [106, 107]. In line with this result, vaccination with recombinant AH1 peptide alone would protect BALB/c mice against CT26 tumor challenge [108]. Moreover, gp70-pulsed DCs efficiently inhibit CT26 lung metastases [109]. Other studies since followed and showed how different adjuvant factors such as Calreticulin [110], GM-CSF [111], IL-21 [112], and HSPs [113] in combination with gp70 play a stimulatory role in CT26 immunogenicity.

Cancer cells are also known producers of interferons, and the expression of IFN-γ has been shown to upregulate MHC class I molecules while downregulating immunosuppressive components, thereby contributing to an effective antitumor immune response [114]. In CT26 cells, however, IFN-γ was shown to promote tumorigenesis despite the upregulation of MHC class I in the tumor cells, and this tumor immune escape was driven by the downregulation of gp70 [106]. Of note, type I IFNs also play a crucial role on APCs, where they are produced by stimulator of interferon genes complex (STING) in response to the cyclic GMP-AMP synthase cytosolic DNA sensing pathway [115], e.g., in response to phagocytosis of a dying tumor cell. Furthermore, in a therapeutic setting, STING expression in tumors of 4T1, CT26, or B16 cells resulted in tumor regression and T-cell priming and memory [116].

The use of AH1 mimotopes—nonapeptide analogs that enhance the expansion and function of TAA-specific T cells upon vaccination [117, 118]—has revealed novel immunogenic features related to AH1 and AH1-expressing cancer cell lines. AH1 and its mimotopes were shown to generate efficient CTL immune responses against established CT26 tumors, even when the epitope-reactive CTLs expressed the immune checkpoint protein programmed cell death 1, which would normally induce CTL anergy [119]. The same group also showed that using AH1 mimotopes induce more cross-reactive anti-AH1 tumor-specific CTLs and stronger anti-tumor protection than AH1 itself in a prophylactic tumor vaccination setting [120, 121].

As touched upon earlier, the CT26 colon carcinoma cell line presents itself with both immunostimulatory as well as immunosuppressive features. While it was shown that 22 murine cell lines of different histological origin all express the MuLV gp70 transcript [98], the colon carcinoma CT26 cell line was characterized to belong to a group of highly immunogenic cell lines based on the significantly greater presence of T-cell co-stimulatory molecules like CD80, CD40, but remarkably also immunosuppressive factors such as TGF-beta and IL-10 in the tumor microenvironment [122]. The opposing expression of both immunostimulatory and immunosuppressive molecules in the tumor microenvironment is intriguing and raises the question of how different immunogenic and non-ICD modalities would affect this immune response balance. In addition to the retroviral antigens expressed by many of the cancer cell lines used for ICD studies, also checkpoint inhibitor ligands such as programmed death-ligand 1 (PD-L1) are present on the cancer cell surface, resulting in a PD-L1/PD-1-mediated blockade of CTL and NK cytotoxicity, which allows tumor immune escape [123, 124]. Administration of immune checkpoint blockers resulted in a stronger reduction of MC38 than CT26 tumor growth in vivo [124, 125] due to a strong upregulation of PD-L1 expression on MC38 tumors compared to CT26 [126].

Besides the retroviral TSA gp70, CT26 cells express more than 150 different mutated genes that encode neoantigenic peptides that are predicted to bind and hence be presented by MHC molecules [88] and the possible neoepitopes therefore represent potential targets for immunotherapy. The CT26 immunopeptidome paper from 2014 was followed up by another elegant study from the same

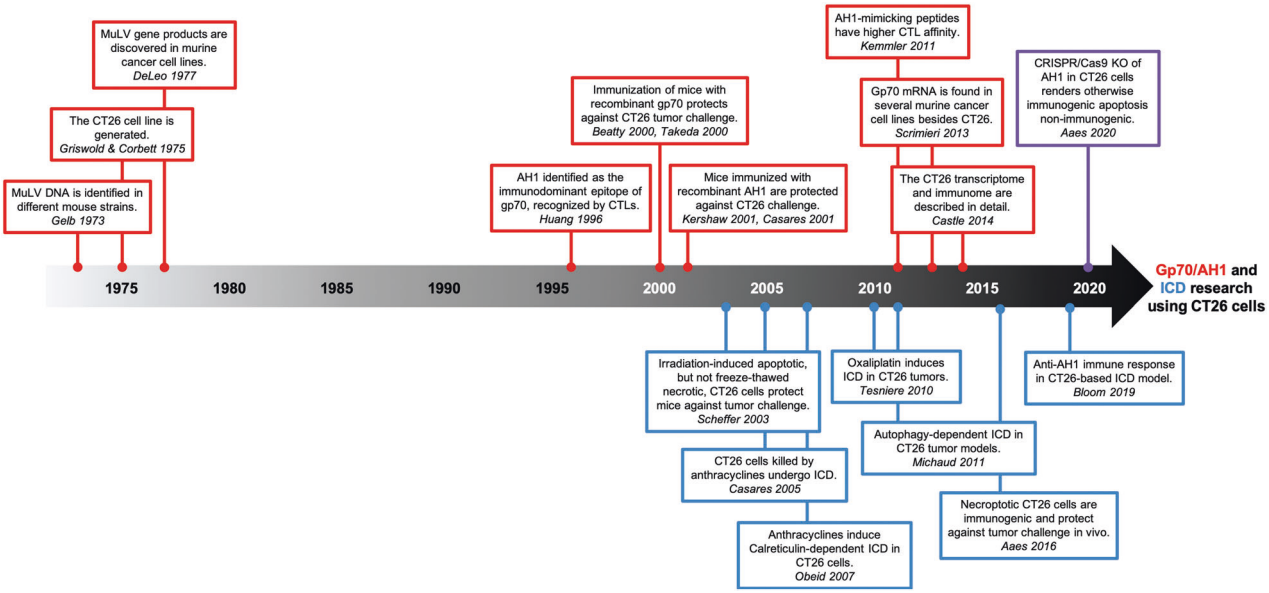

Fig. 2 Timeline showing the occurrence of gp70/AH1-related and ICD research using CT26 cells. The results from selected research papers are highlighted on this timeline, which dates from 1973, when MuLV DNA was first identified in mouse strains. The upper part (in red) highlights the gp70/AH1-centric work and some of the immunogenicity work which was initiated already in the seventies of the twentieth century. The ICD concept was launched only in the early 2000s. On the lower part of the timeline we have pointed out some key papers (in blue), in which CT26 cells were used to define new aspects of ICD. Finally, it was not until this year, 2020, that the immunodominant role of AH1 expression in CT26 cells was directly shown to influence the prototypic apoptotic ICD prophylactic tumor vaccination model (in purple).

research group, in which mRNA encoding a combination of MHC class I and MHC class II epitopes specific to CT26, B16, or 4T1 cancer cells induced potent CTL responses and efficient tumor rejection in syngeneic mice [91]. As the CT26 cells themselves were shown not to express MHC class II molecules, these findings could indicate a role for antigen spread. Antigen spread is a phenomenon in which vaccination against primary TSAs results in the release of secondary (nontargeted) tumor antigens. These secondary antigens prime subsequent immune responses (=antigen spread) [91, 127, 128]. In relation to ICD, two recent papers showed that necroptotic, but not apoptotic, CT26 cells elicit an ICD response toward a mixture of MHC class I and class II neoepitopes [71, 72]. Moreover, we recently showed that an AH1-deficient CT26 cell line, generated by the CRISPR/Cas9 gene-editing technology, lost its ICD potential when undergoing apoptosis, whereas AH1-deficient necroptotic CT26 cells were still able to induce a significant ICD response in vivo [72]. This observation may also be explained by antigen spread, as the antitumor immune response was elicited in vivo in the absence of AH1 in the prophylactic necroptotic tumor vaccination, but yet generated a broad enough immunogenic response to prevent tumor formation upon challenge with live AH1-expressing CT26 and 4T1 cells (Fig. 3). Another explanation could be that necroptotic cell death followed by efferocytosis generates a richer immunopeptidome due to differential proteolysis during necroptosis as compared to apoptosis.

Since the first discovery of MuLV genes in mouse strains [102], numerous reports revealed the immunogenic role of gp70, and later the immunodominant role of the AH1 epitope [97]. Altogether, these findings emphasize the importance of such virally derived TAAs especially when MuLV gene-expressing cell lines are used in immunological studies. Meanwhile, the concept of ICD in cancer research emerged [129], and CT26 became one of the main cell lines to study ICD. Although much of the data covering AH1 and its immunodominant role in CT26 cells were already known, its potential importance for ICD studies seemed rather neglected by the ICD community, up until now, when a direct impact of AH1 expression in CT26 cells on ICD was shown in an antitumor vaccination study [72]. Hence, the early advancement in research covering the immunodominant role of gp70/AH1 in murine cancer cell lines, and the more recent progress in ICD cancer studies using the same gp70-expressing cell lines can be seen as two separate time courses, which only joined paths this year when the AH1-KO CT26 cell lines was used in ICD studies and tumor vaccination models in vivo (Fig. 2). While it is now clearer than ever that AH1 plays an immunodominant role in CT26 tumor vaccination models, we believe that it is of pivotal importance to reconsider the choice and usage of cancer cell lines in future ICD studies and to study the immunogenicity against less immunodominant TAAs such as other potential antigenic epitopes encoded by gp70.

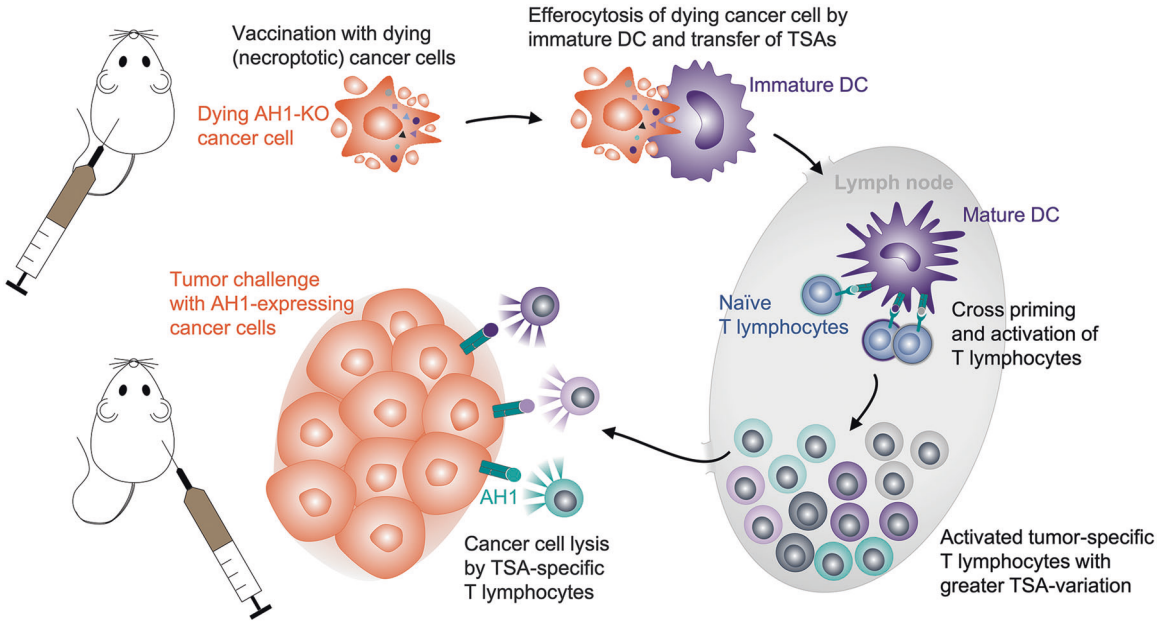

Fig. 3 A role for antigen spread in the prophylactic tumor vaccination model. Following prophylactic vaccination of mice with necroptotic AH1-deficient CT26 cells, the dying CT26 cancer cells are engulfed by dendritic cells during the process of efferocytosis and a transfer of TSAs to the immature DC takes place. In the lymph node, the now mature DCs cross-prime and activate T lymphocytes. Activated T cells kill tumor cells based on specific antigen recognition leading to the release of additional TSAs leading to additional specific T cells. Due to these cycles of antigen spread, a range of activated T lymphocytes with a greater TSA specificity than the original vaccination is generated. Of this pool of TSA-specific CTLs, some are likely to respond to AH1 expression on cancer cells during the challenge phase, and thereby prevent tumor outgrowth.

Perspectives for ICD research

Studying ICD in murine cancer models in the absence of gp70-derived TAs

By definition, an adaptive immune response depends on the recognition of tumor antigens and depending on the type of cancer, different TAAs or TSAs have been identified [130]. Previous studies have revealed the endogenously expressed retroviral tumor antigen in CT26 cells (gp70) and its immunodominant epitope (AH1), which is recognized by CTLs in a context of MHC class I [97]. The immunogenic role of AH1 has been further substantiated in different vaccination studies using recombinant AH1, or AH1 mimotopes [108, 109, 121], and gp70 expression is not confined to CT26 cells only, but importantly is also found in multiple other widely studied murine cell lines such as EL4 lymphoma and B16 melanoma [98, 103, 104]. Consolidating these findings with the immunogenic observations using CT26 cells in the prophylactic tumor vaccine led us to hypothesize that the high expression of the immunodominant tumor antigen epitope AH1 is probably indispensable for the immunogenicity of apoptotic CT26 cells [72]. This was demonstrated in the prophylactic tumor vaccination model using AH1 knockout CT26 cells, generated by the CRISPR/Cas9 gene-editing tool. A subcutaneous injection with apoptotic AH1-deficient CT26 cells was no longer sufficient to establish an antitumor immune protection, and thus demonstrated, in a direct way, the contribution of this single endogenously expressed TAA to the immunogenicity of CT26 cells. To further evaluate the immunodominant role of AH1, we also observed that prophylactic vaccination with necroptotic, but not apoptotic, AH1-deficient CT26 cells still protected against a challenge with another AH1-expressing cancer cell line, 4T1 mammary carcinoma cells, thereby reinforcing the concept that necroptotic vaccination elicited a stronger AH1-independent immune response that even goes beyond the identical tumor challenge. This suggests that other TAAs could be shared between CT26 and 4T1 cells or that certain TAAs are revealed only during necroptotic cell death induction—perhaps an indication of the involvement of antigen spread in this ICD setting.

The observation that immunogenicity and antitumor protection is still achieved in the absence of AH1 expression upon vaccination of mice with necroptotic cells gives us the reason to believe that an adaptive immune response is generated against other tumor antigenic epitopes present in the CT26 cells, because immunogenicity by definition implies the presence of an antigen. As the AH1 peptide sequence (SPSYVYHQF) corresponds to only about 1 kDa, the remaining part of the in total 70 kDa gene product, gp70, is likely to encode other epitopes that could be presented on MHC class I molecules and hence be recognized

by CTLs. In C57BL/6 mice and its derived cancer cell lines MC38 and B16, the other gene product, p15E, deriving from the gp70-precursor protein, has been shown to also give rise to antigen-specific CTLs [99, 108, 131]. Thus, it would also be interesting to investigate the antigenic role of p15E in the setting of CT26 immunogenicity—especially in the absence of the immunodominant expression of AH1. This leads to another subject of interest, concerning the effect of prophylactic vaccination with necroptotic cells that lack the entire gp70 gene product, and identifying which neoepitopes and alternative tumor antigens in CT26 cells would be implicated in response to different cell death mechanisms.

The use of immunogenic cell lines in ICD research probably requires revised experimental models in vivo

Previously, scientists within the ICD field agreed that ICD mechanisms and potential new ICD inducers should be evaluated according to a list of specifically defined criteria in vitro and in vivo [63]. Among the most important in vitro criteria were the detection of DAMPs, and especially in form of (1) CRT exposure on the still intact plasma membrane, (2) ATP secretion from the dying cell, and (3) release of HMGB1. The important contribution of each of these DAMPs has been demonstrated in mouse models, where their blocking or depletion resulted in a marked decrease in immunogenicity of cell death, and in a corresponding manner, reconstitution with each of these DAMPs can bring about an immunological response from otherwise non-ICD inducing conditions [27, 38, 132, 133].

However, there is now reason to wonder whether the prophylactic tumor vaccination model using dead cancer cells should still be regarded as "a gold-standard approach to detect ICD" [63, 134]. Independent studies have demonstrated the intrinsic immunogenicity of various murine cancer cell lines—many of which are routinely used to study ICD. Common to these studies is, for example, immunoprofiling, which shows that some of these cell lines express co-stimulatory factors (e.g., CD80 and MHC class I) that render the cells more immunostimulatory [93, 122, 135]. Studying ICD by using cell lines, which are already highly immunogenic on their own or in which OVA has been introduced, obviously creates a problem—especially when it comes to the prophylactic tumor vaccination model. The chance is that the immunogenic properties of the cell line, even in the absence of any cell death or ICD stimulus, are able to induce an antitumor immune response, which makes it difficult to truly evaluate the immunogenicity of the ICD inducer or the cell death modality in question. Besides the surface expression of immune molecules on cancer cells, the "hot" and "cold" tumor microenvironments are also defined by the tumor cytokine and chemokine production as exemplified by the transcriptional control of NF-κB activated under immunogenic necroptotic conditions [68]. Furthermore, since most of the murine cancer cell lines that are already in use in ICD studies express endogenous retroviral antigens, we would encourage the detection of such non-immunodominant antigenic epitopes in the studies—albeit the cross-priming results may be weaker than what you would achieve with a MA like OVA or bovine serum albumin, this will provide a more clinically relevant tumor antigenic readout.

The observed immunogenic phenotype in response to a dead-cell vaccine will be challenging to evaluate if the cell line even in the absence of an ICD stimulus is capable of activating an adaptive immune response in the host (e.g., by the presence of an immunodominant antigen and the production of cytokines). Therefore, we propose a revised work flow when studying immunogenicity working with murine cancer cell lines (Fig. 4). The first step involves an immunoprofiling of the cancer cell line(s) in question and could include gene expression profiling of immunomodulatory factors in the tumor itself, as well as in draining lymph nodes following tumor inoculation [136]. In case the cancer cell line of interest expresses known endogenous antigens, the immunoprofile could also involve a screening for tumor antigen-responsive CTLs by using epitope-specific tetramer staining on flow cytometry or by IFN-gamma release in ELISpot assays (Fig. 4, Step 1). And for the further use in therapeutic and prophylactic tumor experiments, we would advise to test the minimal amount of injected untreated cancer cells needed to establish a tumor and the amount needed to reject a subsequent tumor challenge (Fig. 4, Step 2).

Alternatively, instead of using the prophylactic tumor vaccination model, immunogenic cell lines should preferentially be used in an experimental therapeutic model that better represents an in situ tumor treatment, which consists of tumor establishment followed by cell death induction. In such an experimental therapeutic model, the typical readout is tumor growth reduction in the presence or not of a cell death stimulus. As an addition to this model, it would be interesting to isolate the tumors and analyze the recruitment of infiltrating immune cells such as antigen-specific CTLs in response to an ICD stimulus versus a control treatment. The basal level of immune infiltration in a tumor established with an immunogenic cell line is based on the vehicle treatment, which serves as a reference level for the amount of immunes cell attraction and activation in the absence of any ICD induction. Due to the inherent immunogenic level of the cell line, it is conceivable that this basal level is higher than in that of a non-immunogenic cell line, and hence, the

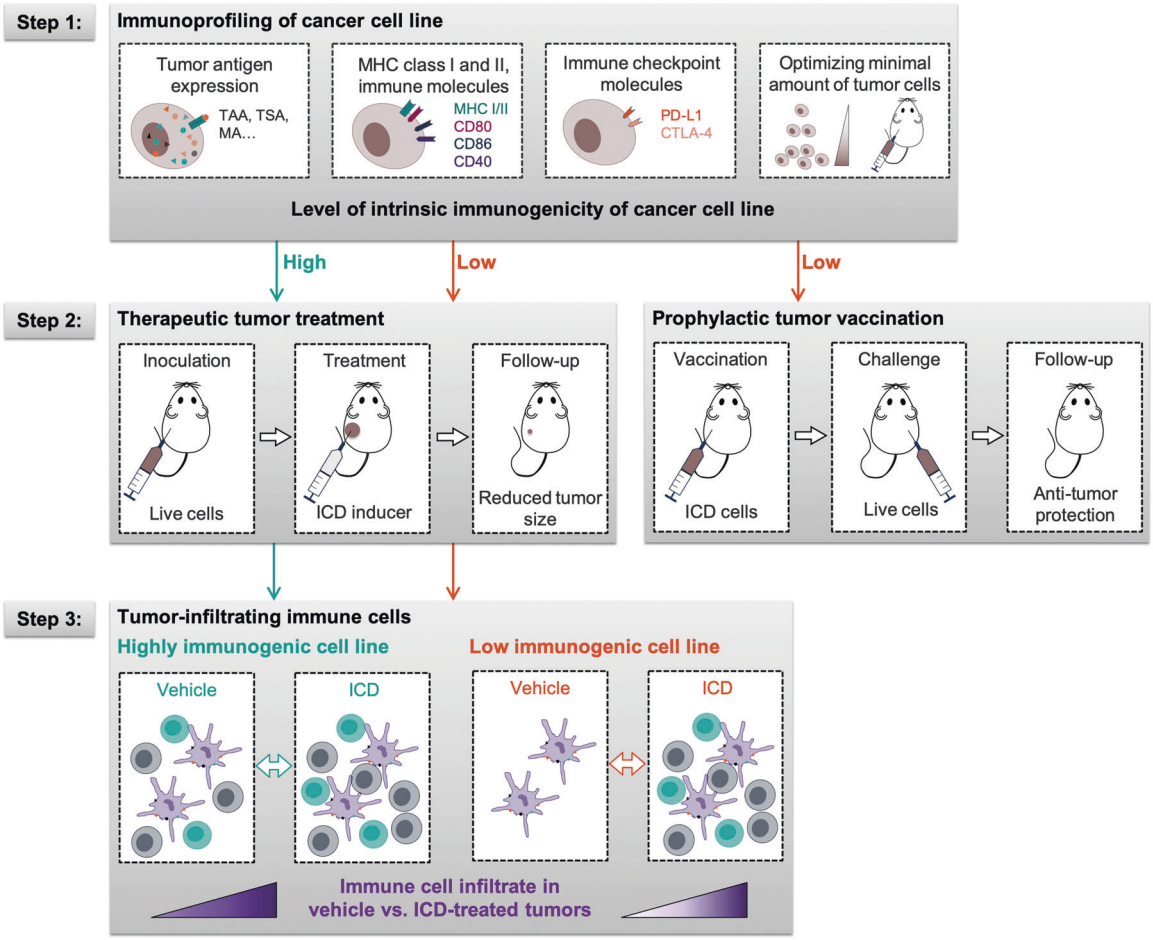

Fig. 4 Suggestive flowchart for the use of murine cancer cell lines in ICD in vivo studies. When choosing a cancer cell line for the use in ICD assays in vivo, we suggest to first (Step 1) determine the expression levels of, e.g., known endogenous tumor antigens (TAA, such as gp70 and p15E retroviral antigens), MHC classes I and II molecule and immunostimulatory and immunoinhibitory molecules. Ideally, the cell lines should also first be tested in a titration vaccination setup using viable untreated cancer cells, before they are applied in an ICD vaccination experiment. Based on these expression profiles, cancer cell lines can be ranked according to their immunoprofile into those cell lines that are likely to be highly immunogenic (cyan arrows) versus low immunogenic (orange arrows). Whereas both high and low immunogenic cell lines are suitable for therapeutic tumor treatment models (Step 2), we would advise that the prophylactic tumor vaccination model is restricted to low immunogenic cell lines. Finally, when studying the level of tumor-infiltrating immune cells (Step 3), the difference between immune cells infiltrates in vehicle- versus ICD-treated tumors may vary between high and low immunogenic cell lines (gradient indicator shown in purple). In tumors established with a highly immunogenic cell line, even in the vehicle-treated background setting, there is likely to be a higher degree of immune cell infiltration than in a corresponding vehicle-treated low immunogenic tumor. Consequently, the relative difference is less pronounced between the amount of infiltrating immune cells in vehicle- and ICD-treated highly immunogenic tumors. On the other hand, in a tumor established with a low immunogenic cell line, we do not expect much if any immune cell infiltrate in a vehicle-treated setting, but once triggered with an ICD inducer, there would be a vast increase in immune cells resulting in relatively high immunogenicity following cell death treatment.

observed increase in infiltrating immune cells upon ICD induction is likely to be less pronounced in the case of the immunogenic cell line relative to that of the non-immunogenic cell line before and after ICD induction (Fig. 4, Step 3).

Tumor antigens in humanized mouse cancer models

As an alternative to mouse-derived cancer cell lines, more therapeutically relevant cancer mouse models use either human-derived cancer cell lines or patient-derived xenografts (PDXs) in immune-deficient mice. However, these models are unsuitable for studying tumor immunology or ICD drugs, due to the lack of host immunosurveillance. Current models are working on engrafting not only PDXs but also matched patient-derived peripheral blood mononuclear cells or humanized $CD34^{+}$ mice [137]. In order to evaluate immunogenic versus non-immunogenic tumor therapies and vaccinations, syngeneic mouse cancer cell line models remain the best option. A comparison of the different types of tumor models—human and mice—currently being tested in the fields of tumor immunology and

immunotherapy has recently also been reviewed elsewhere [138, 139].

Genetically engineered mouse models (GEMMs), or oncomice, were introduced in the 1980s by some of the pioneering work on spontaneous brain tumor models by Richard Palmiter and Ralph Brinster and has since been followed by many others and in many other mouse tissues such as in the pancreas, bones, breast, and skin [140]. The advantages of using GEMMs are that the tumors develop in a natural microenvironment and that they often mimic the histopathological and molecular features of their human counterparts [141]. Just like in patients, the tumors arising in individual GEMM mice will each display unique tumor antigens, and hence the responses to anticancer therapies will be heterogenous. Another disadvantage of using GEMMs in ICD studies is that the antigens which are recognized by CTLs are unknown in most GEMM-derived tumors. This issue can be circumvented by introducing known traceable tumor antigens by, e.g., genetic engineering [141, 142].

Conclusion

In order to understand the true impact of ICD inducers on the adaptive immune response to cancer treatment, we have highlighted some important research articles that have contributed to this field. By consolidating these findings with the vast amount of knowledge on viral-derived endogenous tumor antigen expression in the same ICD-relevant cancer cell lines, we hope to call attention to the unneglectable component of the presence of immunodominant TAAs in ICD cancer research such as retroviral antigens. We propose for dedicated researchers to reevaluate their future choice of especially in vivo ICD detection methods, and instead to base their experimental models on the carefully evaluated immunogenic properties of the cancer cell line in question. As presented in this review, such reevaluation and testing of immunogenic properties could include: endogenous tumor antigen expression levels and recognition by CTLs, surface exposure of MHC molecules, immunostimulatory and immunoinhibitory molecules, checkpoint inhibitor ligand expression, and diluting the cancer cell numbers required for tumor inoculation and rejection in vivo. The results of such immunological assessment would likely lead to an altered choice of experimental therapeutic tumor models compared to prophylactic vaccination models in vivo, and more TSA-directed, including TNAs, experimental setups ex vivo.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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