

## Tumor-intrinsic determinants of immunogenic cell death modalities

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### ABSTRACT

The immune system can recognize tumor cells to mount antigen-specific T cell response. Central to the establishment of T cell-mediated adaptive immunity are the inflammatory events that facilitate antigen presentation by stimulating the expression of MHC and costimulatory molecules and the secretion of pro-inflammatory cytokines. Such inflammatory events can be triggered upon cytotoxic treatments that induce immunogenic cancer cell death modalities. However, cancers have acquired a plethora of mechanisms to subvert, or to hide from, host-encoded immunosurveillance. Here, we discuss how tumor intrinsic oncogenic factors subvert desirable intratumoral inflammation by suppressing immunogenic cell death.

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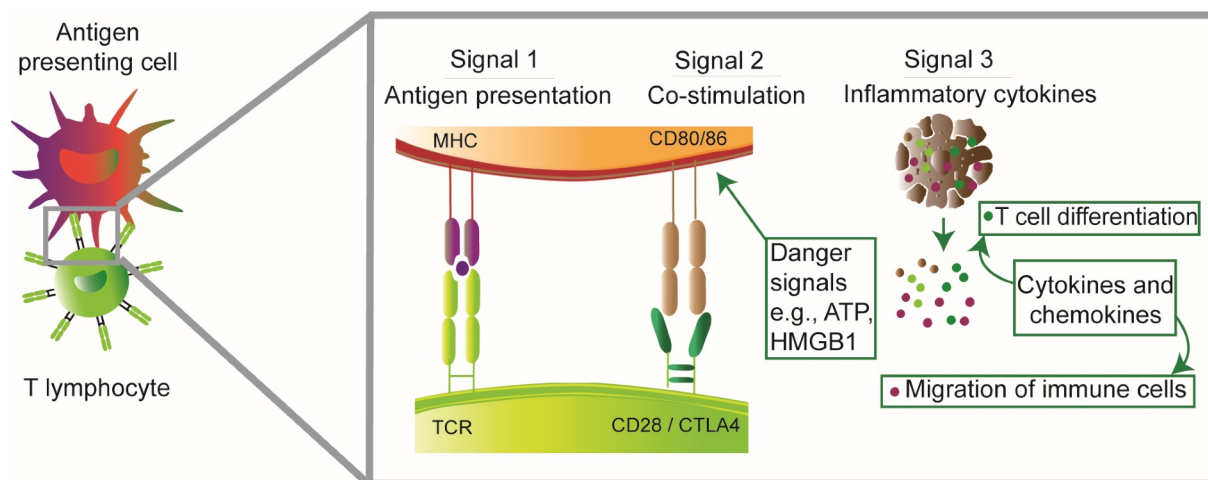
Regulated cell death;  
immunogenic cell death;  
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### Introduction

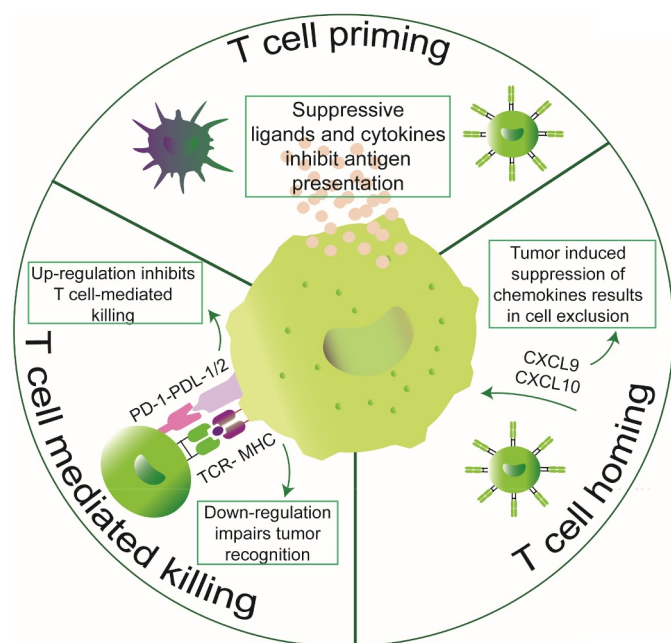
The tumor microenvironment comprises malignant cells, immune cells (innate and adaptive), structural cells (e.g. fibroblasts), vascular cells and others. These cellular constituents function as ecosystems in thus far that they exhibit cooperation (e.g. reciprocal provision of growth factors), competition (e.g. for oxygen, nutrients), predation (e.g. antitumor immunity), and co-evolution in response to selective pressure. The immune system exerts complex tasks to eliminate growing tumors. Innate immune cells such as natural killer (NK) cells are the first to encounter and fight off cancer and, if tumors are not eliminated, the adaptive immune system takes over to mount tumor-specific response by engaging cytotoxic T lymphocytes (CTLs) which are endowed with receptors that recognize tumor antigens presented in the context of major histocompatibility complex (MHC) (Figure 1). Cancer immunoediting is the process whereby the immune system can both constrain and promote tumor growth. The concept of cancer immunoediting relies on sequential stages, namely elimination, equilibrium and escape, that depict the interaction of growing tumors with the immune system<sup>1</sup>. Over-expressed tumor-associated self-antigens, non-self-mutated neoantigens and oncogenic virus-associated epitopes can be cross-presented to naïve T cells and activate tumor-specific CTL responses to eliminate immunogenic tumors<sup>1</sup>. The immune pressure added to genomic instability provides tumors an opportunity to develop malignant escape variants that undergo progressive selection and outgrowth. Thus, clinically diagnosed macroscopic tumors are usually

immune-edited and have defeated host intrinsic immunosurveillance mechanisms (Figure 2).

Several immunotherapeutic approaches can reactivate pre-existing immune infiltrates to provide long-term survival of cancer patients. Moreover, in tumors that lack immune infiltrates induction of inflammatory cell death can kickstart the anticancer immunity cycle and reinstate immunosurveillance (Figure 3).<sup>2</sup> In fact, certain types of cytotoxic chemotherapies<sup>3</sup> or targeted therapies such as radiation,<sup>4</sup> oncolytic viruses,<sup>5</sup> photodynamic therapy,<sup>6</sup> extracorporeal photochemotherapy<sup>7,8</sup> can activate tumor cell stress and immunogenic cell death (ICD) that positively contributes to immune-mediated recognition of tumors<sup>3</sup> (Figure 3). Depending on the cytotoxic stimuli, ICD consists of a cascade of events that starts with a premortem stress and leads to a cellular demise that concurrently allows the release of immunomodulatory molecules and tumor cell contents. In these scenarios, danger molecules and cytokines released during ICD are critical for successful tumor-antigen presentation and development of adaptive immunity. Thus, approaches that combine ICD inducers with immune checkpoint inhibitors<sup>2</sup> that block inhibitory T cell receptors<sup>9,10</sup> have gained clinical popularity.<sup>11,12</sup> However, emerging studies show direct evidence that tumor-intrinsic oncogenic factors can influence the immunogenicity of tumor cell death<sup>13,14</sup> (Figure 4). Here we review recent advances as to how tumor-specific oncogenic factors (oncogenes and tumor suppressors) influence the anticancer immunity cycle by dictating the activation of inflammatory cell death as well as the secretion of immunomodulatory molecules during ICD. We conclude by illustrating how knowledge of tumor genetics can be integrated into patient



**Figure 1.** Mechanisms of tumor antigen presentation. Antigen-presenting cells (APCs) deliver peptides via major histocompatibility complex (MHC) to the T cell receptor (TCR). This initial peptide-loaded MHC-TCR interaction signal activates the T cell, but for antigen presentation to succeed, two additional steps are required. Engagement of co-stimulatory and co-inhibitory molecules is the second signal that determines T cell function and fate by promoting or inhibiting T cell activation and function. The third signal required for successful antigen presentation is the provision of an inflammatory cytokine milieu that determines the differentiation of T cells. ICD stimulates antigen presentation at signals two and three by releasing danger molecules that increase co-stimulatory molecule expression and providing cytokines essential for T cell differentiation.



**Figure 2.** Mechanisms of tumor-mediated immune evasion. Tumors can inhibit intrinsically developed immunity at the three stages of anticancer immunity cycle. Tumors secrete suppressive cytokines and ligands to inhibit T cell priming. Moreover, tumors can also conspire the secretion of chemokines that are essential for entry of cytotoxic T lymphocytes into the tumor stroma. Lastly, tumors can also inhibit T cell-mediated killing either by downregulating components of the antigen processing and presentation machinery or by expressing T cell inhibitory ligands such as PD-L1/2.

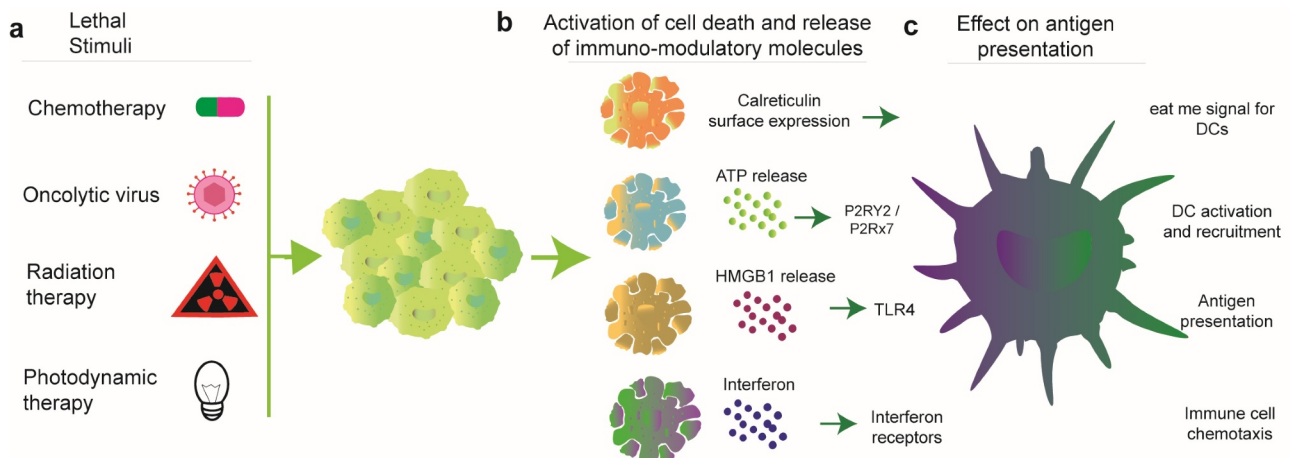
selection for maximizing immunotherapeutic outcome after ICD-inducing treatments.

### Mechanisms of antitumor immunity after ICD

Mechanisms of ICD and downstream events leading to tumor-specific CTL response have been mostly investigated in the context of cancer chemotherapy. In this setting, stressed and

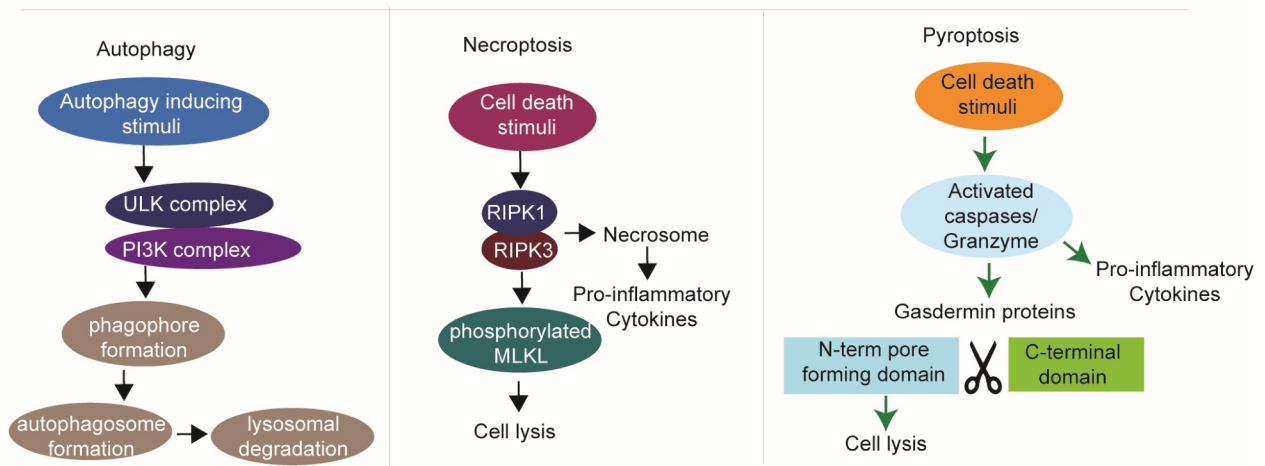
dying malignant cells display or release Danger Associated Molecular Patterns (DAMPs) that attract and activate proficient antigen-presenting cells, mainly immature dendritic cells (DCs). Among these alarm signals are (1) the surface exposure of the endoplasmic reticulum chaperone calreticulin (CALR) and the release into the tumor microenvironment (TME) of (2) the cytoplasmic protein annexin A1 (ANXA1), (3) the nucleotide ATP, and (4) the nonhistone chromatin-binding protein high-molecular group B1 (HMGB1) (Figure 3). These DAMPs are sensed by DCs through the low-density lipoprotein receptor-related protein 1 (LRP1, also known as CD91), formyl peptide receptor-1 (FPR1), purinergic receptors P2RX7 and P2RY2, and TLR4, respectively. Additionally, treatment by ICD chemo-inducers mimics viral infection and stimulates the secretion by malignant entities of type I interferons (IFN) and C-X-C motif chemokine ligand 10 (CXCL10), thus attracting T lymphocytes. Once at proximity of DAMP-emitting dying cancer cells, DCs will experience activation and maturation which implies up-regulation of the lymphoid tissue-residing C-C motif chemokine receptor 7 (CCR7), of MHC molecules and co-stimulatory factors (e.g. CD80, CD86), as well as the production of inflammatory cytokines (e.g. interleukin-12 [IL12], IL6, tumor necrosis factor-alpha [TNF- $\alpha$ ]).

DCs are able to engulf tumor antigens notably via phagocytosis of malignant cell corpses, or via macropinocytosis of free antigens that may have spread upon cell death.<sup>15</sup> Then, DCs process captured antigens and proceed to cross-presentation of associated epitopes onto MHC (Figure 1). Of note, DC populations that appeared particularly enriched in the tumor bed and seemed predictive of an improved cancer outcome consist of Ly6C<sup>hi</sup>CD11b<sup>+</sup> monocyte-derived DCs and of Clec9A<sup>+</sup> type 1 conventional DCs, particularly the CD103<sup>+</sup> subset.<sup>16</sup> Recently, a platelet factor P-selectin was shown to initiate cross-presentation.<sup>17</sup> Mature DCs migrate to secondary lymphoid organs to prime cognate naive CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes.<sup>18</sup> This step not only requires the interaction of the MHC/epitope complex with the T cell receptor (TCR)

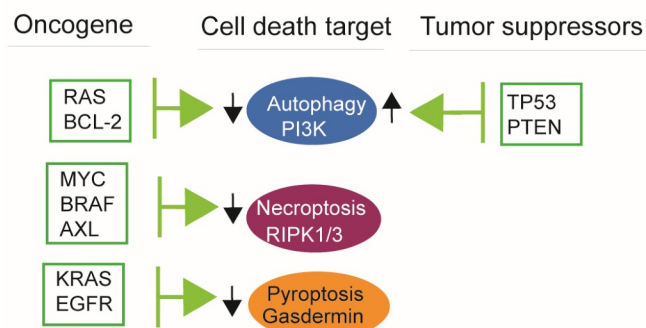


**Figure 3. Mechanisms of ICD.** Several types of lethal stimuli (a) activate tumor cell stress and cell death that leads to the surface expression of the “eat me” signal calreticulin and extracellular release of ATP, HMGB1 and interferons (b). In a concerted effort, the danger molecules released during ICD promote antigen presentation and immune cell trafficking (c).

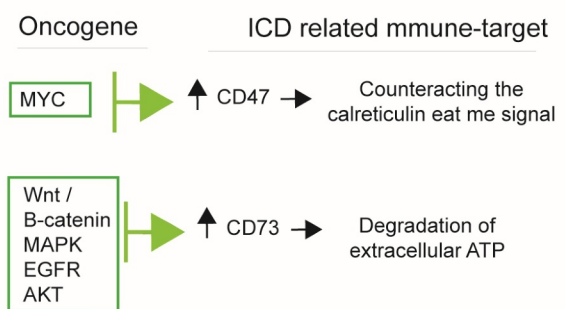
**a Signaling pathways of inflammatory cell death**



**b Modulation of cell death by tumor intrinsic factors**



**c Oncogenic factors abolish the activity of DAMPs**



**Figure 4. Tumor intrinsic oncogenic factors dictate the activation and execution of ICD.** (a) Diverse genotoxic and metabolic stimuli initiate signaling pathways to activate autophagy, necroptosis and pyroptosis. (b) All these three types of inflammatory cell death can be modulated by tumor intrinsic factors. (c) Several types of oncogenes evade ICD by indirectly activating immune-suppressive ligands.

but also a co-stimulatory signal resulting from the binding of DC surface-exposed CD80/CD86 to T cell surface-exposed CD28, as well as the secretion of some cytokines supporting T cell differentiation and proliferation such as IL2<sup>19</sup>(Figure 1). Activated type 1 helper CD4<sup>+</sup> T (Th1) and cytotoxic CD8<sup>+</sup>

T (Tc1) lymphocytes also produce large amounts of IFN $\gamma$ , which exhibits pleiotropic antitumor activity. First, IFN $\gamma$  maintains Th1 T cell lineage commitment and stimulates differentiation of CD8<sup>+</sup> T cells into CTLs (positive feedback loop), boosts antigenic exposure in antigen-presenting cells and

target cells, and favors macrophage activation and polarization toward a tumoricidal phenotype (e.g. enhanced phagocytic potential, production of nitric oxide, tryptophan depletion)<sup>20</sup>. Second, IFN $\gamma$  can exert direct anti-proliferative (e.g. regulation of p21 expression), anti-angiogenic (e.g. impaired survival of endothelial cells) and pro-apoptotic/necroptotic (e.g. up-regulation of caspases, enhanced secretion of Fas and Fas ligand) effects on transformed cells.<sup>20</sup> Additionally, activated T lymphocytes produce TNF $\alpha$  which cooperates with IFN $\gamma$  to further stimulate Th1/Tc1 responses and to sensitize cancer cells to apoptosis (e.g. repression of B-cell lymphoma-extra large [Bcl-xL] expression, ischemia via endothelial cell apoptosis).<sup>20,21</sup> Upon activation, effector T cells, notably CTLs, express C-X-C motif chemokine receptor 3 (CXCR3) that will allow their migration to the tumor bed in a, yet poorly understood, paracrine CXCL9/CXCL10/CXCL11-dependent manner<sup>22</sup> (Figure 2). These chemokines are mostly secreted by cancer cells, monocytes, endothelial cells and fibroblasts in response to IFN- $\gamma$ .<sup>22</sup> Then malignant cells harboring MHC-I-coupled epitopes can be targeted by cognate CTLs which achieve their antitumor function mainly through the secretion of perforin, a pore-forming toxin triggering osmotic lysis.<sup>23,24</sup> Collectively, this series of events has been termed “cancer-immunity cycle” by Dan Chen and Ira Mellman back in 2013<sup>18</sup> (Figure 2).

### **Adaptive immune-resistance mechanisms encoded by tumors**

Cancer cells adapt to overcome the aforementioned immunosurveillance mechanisms and resist to immune attack. These pro-tumoral immune evasion actions mostly prevent either the ignition of the cancer-immunity cycle at the level of antigen presentation by inhibiting DC function, or terminally suppress the effector function of cytotoxic T lymphocytes<sup>25</sup> (Figure 2). First, immunoediting tends to select poorly immunogenic transformed cells. Hiding from the adaptive immune cell radar can be achieved through mutations or epigenetic silencing of relevant tumor antigens or through a general reduction of antigen presentation, via the loss of class-I MHC, beta-2-microglobulin (B2M) or antigen peptide transporters 1 and 2 (TAP1/2).<sup>26,27</sup> Alternatively, some malignant cells constitutively expose inhibitory immune checkpoints such as programmed death-ligand 1 (PD-L1)<sup>28</sup> or secrete immunosuppressive factors like indoleamine 2, 3-dioxygenase 1 (IDO1).<sup>29</sup> Both PD-L1 and IDO1 are normally stimulated by IFN- $\gamma$  as a negative feedback loop.<sup>29–31</sup> PD-L1 interacts with the activation/exhaustion marker programmed cell death 1 (PD-1) on tumor-infiltrating T lymphocytes (TILs) and impair cytokine production as well as T cell proliferation and survival.<sup>32</sup> Released IDO1 depletes tryptophan (Trp) from the extracellular milieu thus promoting cell cycle arrest and death of effector TILs.<sup>29</sup> Furthermore, Trp catabolism by IDO1 enzymatic activity generates kynurenine (Kyn). The latter can bind the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, at the surface of DCs and regulatory CD4<sup>+</sup> T lymphocytes (Tregs). In DCs, Kyn-activated AhR triggers the release of immunosuppressive IL10 while inhibiting the production of immunostimulatory IFN $\beta$ . Meanwhile, nuclear

translocation of activated AhR in immunosuppressive Tregs stimulates their proliferation within the TME.<sup>29</sup> In addition to IFN-induced adaptive immune resistance, cancer cells also acquire resistance to cancer immunosurveillance and immunotherapy via inflammatory cytokine- and stress-related mechanisms.<sup>33–36</sup> For instance, in a preclinical murine model of melanoma, the release of TNF $\alpha$  by TILs initiated dedifferentiation of melanoma cells and ultimately translated into a loss of several melanosomal antigens. As a consequence, a therapy relying on the adoptive transfer of T cells specific for the melanoma-associated antigen gp100 only showed transient responses.<sup>33,34</sup> Oncogenic pathways that support the abovementioned mechanisms of adaptive immune resistance remain poorly documented and will be introduced in the following paragraph.<sup>37</sup>

### **Oncogenic driver mutations influence cancer immune contexture and immunosurveillance**

Genomic landscapes of common human cancers revealed around 140 genes that can drive tumorigenesis when altered by intragenic mutations.<sup>38</sup> Common to most solid tumors is that the major driver oncogenic mutations confer selective growth and proliferative advantage by altering up to 12 cancer cell-intrinsic cellular signaling pathways.<sup>38</sup> These deregulated pathways confer distinct and complementary capabilities that confer on tumor cells a selective growth advantage by increasing cell division and preventing cell death.<sup>39</sup> In parallel, cancer cells must acquire the ability to prevent immune cell recognition and elimination.<sup>40</sup> The composition and function of immune cells in tumors vary greatly between and within cancer histotypes.<sup>41,42</sup> This considerable variation in phenotypic and functional characteristics of intratumoral leukocyte composition impact cancer outcome.<sup>43</sup> Accumulating clinical observations and mouse studies reveal that gain-of-function mutations in oncogenes (e.g. KRAS, MYC) and loss-of-function alterations in tumor suppressor genes (e.g. PTEN, TP53) are correlated with changes in immune composition and response to immunotherapy.<sup>44</sup> Overall, mouse and human studies showed that inactivation of tumor suppressors as well as activation of oncogenes directly contribute to pro-tumoral immune contexture and failed immunosurveillance.<sup>44</sup> The mechanisms by which oncogenic driver mutations orchestrate highly immunosuppressive tumor microenvironments are under intense investigation. Pro-oncogenic signals can affect the cancer-immunity cycle at the stages of (1) tumor antigen capture and presentation, (2) T cell activation and recruitment to malignant lesions, and of the (3) tumoricidal activity of T cells within the tumor (Figure 2). This review will document how tumor intrinsic oncogenic events influence ICD and thereby influence tumor antigen presentation.

### **Do tumor-intrinsic oncogenic events affect ICD?**

Over-activated oncogenic signaling at early stages of cellular transformation creates metabolic stress that compel malignant cells to activate programmed cell death.<sup>45</sup> Thus, only malignant cells that acquire additional molecular defects to confer interruption of cell death signaling cascades are able to continue

proliferation.<sup>46,47</sup> In addition to cell-autonomous effects, cell death effectors can exert non-cell-autonomous effects by regulating the release of danger molecules and cytokines into the tumor microenvironment.<sup>48–50</sup> Thus, tumors manipulate programmed cell death to evade immunosurveillance. Malignant cells suppress programmed cell death signaling through loss-of-function mutations in proteins that sense and transduce lethal signals or execute cell death (as in many tumor suppressor proteins), and also from gain-of-function alterations in oncogenes that normally deliver pro-survival signals.<sup>51,52</sup> Depending on the cytotoxic stimuli, ICD consists of a cascade of events which starts with a premortem stress and leads to a cellular demise that concurrently allows the release of immunomodulatory molecules and tumor cell content. Thus, successful execution of ICD involves three distinct and interlinked biological processes that include (1) activation of premortem stress and execution of inflammatory cell death, (2) expression and/or secretion of immunomodulatory molecules, and (3) exogenous effects of these immunomodulatory molecules which consist in the recruitment and activation of immune cells (Figure 3). In the next section of the review, we provide new insights and mechanisms as to how tumor-specific oncogenic events interfere at the first two steps of ICD activation.

### 1. Effect of tumor-intrinsic oncogenic events on the initiation and execution of inflammatory cell death modalities

It is now widely accepted that oncogenic events, depending on cancer type, select for tumors that initially resist cell death. Due to inactivation of the molecular pathways that sense cellular stress to activate cell death, malignant cells are relatively resistant to death upon chemotherapy and radiotherapy.<sup>53</sup> Although the mechanisms are mostly unknown, tumors dampen signaling pathways required for sensing intracellular and extracellular stress.<sup>54,55</sup> Several sensors of damage-associated molecular patterns (DAMPs), including Toll-like receptors (TLRs),<sup>55</sup> the cytosolic RNA-sensors retinoic acid-inducible gene I (RIG-I)<sup>56</sup> and melanoma differentiation A protein 5 (MDA-5), as well as stimulator of interferon genes (STING),<sup>54</sup> appear down-regulated in multiple cancers compared to healthy tissues. Collectively, these deregulated pathways enable tumor cells to counter immunosurveillance, partly by preventing them to sense stress and to activate immune sentinels. Recent studies showed that oncogenic factors interfere in certain tumor types with the activation and execution of inflammatory cell death by regulating the expression of cell death executioner caspases<sup>57,58</sup> and kinases<sup>14</sup> (Figure 4). Moreover, inhibition of major oncogenes, such as KRAS, causes tumors to die in an immunogenic fashion thereby reinstating immunosurveillance.<sup>13</sup> The following sections of the review will describe how tumor-intrinsic oncogenic factors interfere with ICD by manipulating cell death and survival programs including autophagy, necroptosis and pyroptosis, or again ICD-related hallmarks.

### Autophagy

Autophagy is an evolutionarily conserved lysosomal degradation process that is critical for nutrient recycling and metabolic adaptation during stress (Figure 4a). Thus, autophagy intervenes in a plethora of biological phenomena such as mitochondrial function, stress response, cellular homeostasis, metabolism, cell death and immune surveillance.<sup>59</sup> Autophagy is linked to many types of pathologies including cancer. Autophagy plays a complex role in tumor development and progression by influencing different aspects of the tumor–host interaction. Autophagy constitutes a cell-intrinsic barrier against malignant transformation<sup>60</sup> by activating oncogene-induced cell death,<sup>45</sup> cellular senescence,<sup>61,62</sup> removal of reactive oxygen species which can damage mitochondria,<sup>63</sup> maintenance of genomic stability<sup>64</sup> and degradation of oncogenic proteins.<sup>65–69</sup> Consistent with this notion, deletion of autophagy regulators in tumor-prone mouse results in increased incidence of lung and liver tumors as well as lymphomas.<sup>70–73</sup> However, in established tumors, autophagy is often used as an adaptive mechanism for tumors to thrive in nutrient-depleted and hypoxic tumor microenvironments.<sup>74–77</sup> Although tumor cells vary in their autophagy dependency, inhibiting autophagy in established tumors generally results in dramatic tumor reduction and prolonged survival in murine models.<sup>78,79</sup> In accordance with these observations, advanced human tumors often display enhanced autophagic flux.<sup>80</sup>

The non-cell-autonomous antitumor effects of autophagy are linked to its immunomodulatory role in the immune TME.<sup>12,81–83</sup> Autophagy induction in anthracycline-treated dying tumors facilitates the release of immune-stimulatory ATP to the extracellular microenvironment, thereby potentiating tumor-antigen presentation and immune-mediated recognition of tumors.<sup>81–84</sup> In this line, deletion of ATG5 in KRAS mutant tumors results in accelerated oncogenesis by creating highly immunosuppressive microenvironment associated with Treg-mediated inhibition of cancer immunosurveillance.<sup>78</sup> Moreover, autophagy has been shown to promote cross-priming of tumor-specific CD8<sup>+</sup> T cells.<sup>85</sup> In immune cells, autophagy plays a role in the formation of memory CD8<sup>+</sup> T cells.<sup>86</sup> Overall, these findings suggest that both tumor-intrinsic and systemic host defects in autophagy may prevent the immune system to detect and eliminate pre-malignant and malignant cells. In contrast, tumor cell autophagy has also been shown to inhibit NK cell cytotoxicity in some tumor types.<sup>87</sup>

Due to its tumor cell-autonomous and immune-mediated microenvironmental effects, autophagy is subject to modulation by oncogenic and tumor suppressor proteins<sup>88,89</sup> (Figure 4b). Many oncogenes, such as BCL-2 and RAS directly inactivate components of the autophagy machinery. Several members of the BCL-2 protein family inhibit autophagy by sequestering BECN1.<sup>90–92</sup> Hyperactivated RAS engages the PI3K/PDPK1/AKT1 signaling cascade to potently suppress autophagic responses.<sup>93</sup> Tumor suppressors such as TP53 engage the cell death machinery in tumors that harbor irreparable molecular lesions such as DNA damage. TP53 can promote autophagy by transactivating multiple autophagy-related genes including AMPK and BH3 family of proteins (such as BAD, BNIP3). Moreover, the phosphatase and tensin homolog

protein (PTEN) is a tumor suppressor with autophagy promoting functions by antagonizing PI3K signaling. The transcription factor forkhead box O1 (FOXO1) is a tumor suppressor required for stress-induced autophagy by directly interacting with ATG7. Taken together, tumor suppressors activate autophagy to limit malignant cell progression. Malignant cells that have acquired the hallmarks of inactivating tumor suppressors along with hyperactivated oncogenes will progress to down-regulate autophagy. Additional genetic defects may cooperate with oncogenes and tumor suppressors to regulate autophagy during tumor progression.

### Necroptosis

Necroptosis is a programmed cell death triggered by various stimuli that include engagement of death receptors, IFNs, TLRs, intracellular RNA and DNA sensors, as well as genotoxic and oxidative stresses induced by anticancer drugs<sup>94</sup> (Figure 4a). The role of necroptosis in anticancer immunity remains unclear. Indeed, necroptosis can be pro-tumoral<sup>95</sup> and antitumoral<sup>96–98</sup> depending on the nature of the lethal stimulus and tumor model. On one side, George Miller and colleagues identified that pancreatic tumors have higher basal expression of the necrosome components that is further augmented after treatment with gemcitabine. Impeding necroptosis *in vivo* resulted in a more inflammatory tumor infiltrate associated with elevated CD8<sup>+</sup> T lymphocytes and reduced myeloid-derived suppressor cells. These pro-tumorigenic effects of necroptosis seemed specific to tumors that were growing in the pancreatic microenvironment. Yet, recently in melanoma, an unbiased CRISPR screen identified RIPK1 as a top candidate inhibiting immunotherapy with immune checkpoint inhibitors.<sup>99</sup> On the other side, antitumor effects of necroptosis are based on overexpression of the executioner proteins RIPK3 and MLKL.<sup>96–98</sup> These observations were mainly made in vaccination studies that specifically activate necroptosis in fibroblasts following chemically induced dimerization of RIPK1/3 to activate cytokine secretion and cell lysis. Intratumoral injection of necroptotic fibroblasts provides pro-inflammatory cytokines that serve as adjuvants to activate antitumor immunity in a non-antigen-dependent fashion.<sup>96–98</sup> The paradoxical effects of necroptosis on immune activation may arise from differences in the lethal stimuli and immune responsiveness of the tumor models.

Components of the necroptosis machinery are deregulated in many cancer types (Figure 4b). Tumors display different types of mutations in the proteins that execute necroptosis. Some cancer types shut off necroptosis through (1) genetic and epigenetic down-regulation of RIPK3 and MLKL expression in acute myeloid leukemia, breast, colon and colorectal cancer types,<sup>100–102</sup> and (2) acquired mutations in functional domains of RIPK3 and MLKL that hinder necroptosis signaling or cell lysis during necroptosis.<sup>103,104</sup> In this line, tumor-specific oncogenic events are shown to directly regulate the expression of RIPK1 and RIPK3<sup>14</sup>. The actin crosslinking protein  $\alpha$ -actinin-4 (ACTN4) is emerging as crucial regulator of carcinogenesis. ACTN4 serves as a scaffold to stabilize RIPK1 by allowing association of RIPK1 and cellular inhibitor of apoptosis protein 1 (cIAP1) to activate NF- $\kappa$ B.<sup>105</sup> A recent study on

941 human cancer cell lines came to the conclusion that 83% of the cells are resistant to necroptosis, irrespective of the tissue and cancer subtype. Bioinformatic analyses revealed that 20 oncogenic hits hinder necroptosis by down-regulating RIPK3 expression.<sup>14</sup> Chemical inhibition of the oncogenes AXL (using BMS-777607) and BRAF (TAK-632) up-regulated RIPK3 expression in tumor cells.<sup>14</sup> However, shutting down the necroptosis pathway is not a general mechanism exploited by all types of cancer cells to survive and progress. Indeed, the expression of necroptotic players was found to be elevated in glioblastoma,<sup>106</sup> lung<sup>107</sup> and ovarian cancers.<sup>108</sup> Future studies should explore the genetic and epigenetic interactions of oncogenes and tumor suppressors with the necroptosis machinery in a broad range of cancers.

### Pyroptosis

Pyroptosis is an inflammatory form of programmed necrosis that serves as an immune effector mechanism against microbes and cancer<sup>109</sup> (Figure 4a). A diverse range of ligands and genotoxic stressors stimulate the inflammatory signaling cascade that culminates in the activation of caspases that subsequently cleave and activate gasdermin. Irrespective of the cell death stimuli and signaling cascade, gasdermin cleavage represents a terminal event during pyroptosis.<sup>110</sup> Proteins of the gasdermin family (which consists of gasdermins A, B, C, D, and E as well as of Pejvakin) are expressed in normal tissues<sup>111</sup> in an autoinhibited state (with the exception of Pejvakin). Following caspase- and granzyme A/B-mediated cleavage, the active N-terminal domain of gasdermin binds to the plasma membrane to generate pores that disrupt their barrier function, resulting in cell swelling and eventual lysis.<sup>111</sup> Moreover, the pores serve as gates for the extracellular release of danger signals and cytokines.<sup>111</sup>

The pathophysiological role of pyroptosis in cancer is expanding. Many types of anticancer chemotherapies including topotecan, etoposide, cisplatin, 5-fluorouracil and paclitaxel activate pyroptosis in cancer cell lines in a gasdermin-dependent fashion. Activation of pyroptosis in tumors can exert both tumor-promoting and antitumor immune effects.<sup>112</sup> Pro-tumor effects of pyroptosis are reported for pancreatic tumors and mainly linked to chronic activation of the inflammasome which attracts MDSCs.<sup>113</sup> The tumor-promoting role of inflammasomes is related to immune suppression consecutive to secretion of the cytokines IL-1 $\beta$ <sup>114,115</sup> and IL-18.<sup>116–118</sup> On the contrary, antitumor effects of inflammasomes were witnessed in colorectal cancer. As inflammasomes are major drivers of pyroptosis, the lack of inflammasome mediators in colorectal cancer was associated with pronounced tumor growth.<sup>119–122</sup> In line with the antitumor effects of pyroptosis, loss of gasdermin expression is correlated with aggressive cancers and increased risk of metastasis<sup>123</sup> whereas expression of full-length gasdermin E in mouse cancers stimulate antitumor immunity.<sup>124,125</sup> Two independent groups showed that ectopically expressed full-length gasdermin E is cleaved by granzyme A/B released by cytotoxic T lymphocytes and NK cells, resulting in the release of N-terminal gasdermin that forms pores in cancer cells.<sup>124,125</sup>

Several studies revealed that gasdermin proteins A, C, D and E are down-regulated in human breast,<sup>125</sup> gastric<sup>126</sup> and colorectal cancer cell lines, as well as in primary tumors<sup>126,127</sup> (Figure 4b). Accordingly, targeted therapies like erlotinib and trametinib, which inhibit oncogenic signaling by targeting EGFR and KRAS, respectively, resulted in increased pyroptosis.<sup>128</sup> These results suggest that the oncogenes EGFR and KRAS may suppress pyroptosis. Further research should explore the mechanisms by which tumor-specific oncogenic events downregulate pyroptosis.

## 2. Effect of tumor-intrinsic oncogenic events on the secretion of ICD-induced DAMPs

Sensing of lethal stimuli to activate cellular stress and programmed cell death is essential to enforce tumor cells to emit immunomodulatory molecules (Figure 3). In the last two decades, the molecular mechanisms of ICD-induced immune activation have profoundly enriched our understanding on the immunomodulatory molecules released during cell death<sup>129,130</sup> and how that affects recruitment of immune cells to the tumor bed, innate and adaptive immune cell activation, and anticancer activity.<sup>2,82</sup> Overall, these studies have shown that various forms of ICD inducers promote the exposure or release/secretion of immunomodulatory molecules such as CALR,<sup>131</sup> HMGB1, ATP,<sup>132</sup> type I IFNs,<sup>41</sup> CXCL10,<sup>41</sup> and CXCL1.<sup>42</sup> ICD inherently relies on the ability of cancer cells to display these signals (Figure 3). Yet, tumors vary in their ability to secrete immuno-modulatory molecules upon programmed cell death.<sup>133–135</sup> These differences can be attributed to the origin of the tumor tissue,<sup>136</sup> underlying oncogenic events and (epi)genetic lesions the tumor has accumulated over time. Studies elucidating the direct effects of oncogenic events on the secretion and/or exposure of immunomodulatory molecules are generally lacking. However, a few recent studies have shown that oncogenes<sup>137,138</sup> can influence the expression of immunomodulatory molecules during ICD. In the next sections, we review recent literature describing how oncogenic events can influence the level of immunomodulatory molecules emitted by malignant entities experiencing ICD (Figure 4c).

### Calreticulin (CALR)

CALR is an endoplasmic reticulum resident protein critically important for maintaining calcium homeostasis and serves as a molecular chaperone to prevent the export of misfolded proteins to the Golgi apparatus.<sup>139,140</sup> Calreticulin plays additional immunological functions such as facilitating the phagocytic uptake of dying tumor cells by innate immune cells<sup>129,141</sup> and serving as integral part of the peptide-loading complex for antigen presentation in the context of MHC-I.<sup>142,143</sup> Moreover, CALR has many immune-related functions in T cells that have been extensively reviewed elsewhere.<sup>93</sup> Conversely, high expression of intracellular CALR promotes tumor cell proliferation contributing to metastasis in multiple cancer types.

Contrasting with the pro-tumor impact of intracellular CALR, surface-exposed CALR, following treatment with ICD-inducing chemotherapies, promotes tumor cell uptake by

phagocytic cells.<sup>129,141</sup> There are two ways by which CALR reaches the surface of dying cells. The first pathway relies on transcription and translational inhibition,<sup>144</sup> leading to the phosphorylation of eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) by the ER stress-sensing kinase, PKR-related ER kinase (PERK), and subsequent activation of caspase-8 and BAX. Finally, a pool of CALR that has transited the Golgi apparatus is secreted by SNAP receptor (SNARE)-dependent exocytosis.<sup>145</sup> In addition, paracrine signals that involve the chemokine CXCL8 contribute to CALR exposure in an eIF2 $\alpha$ /PERK/BAX//BAK-dependent fashion.<sup>146</sup> Vaccination of mice with dying tumor cells deficient in any of the proteins required for CALR exposure, or with cells in which CALR was knocked down, reduced the immunogenicity of the dying cell vaccine.<sup>145</sup> The second pathway for CALR exposure is activated by photodynamic therapy and relies on direct PERK-mediated trafficking of CALR by regulation of the proximal secretory pathway.<sup>147</sup> In this signaling pathway, eIF2 $\alpha$  phosphorylation and caspase-8 signaling are dispensable for CALR exposure.<sup>147</sup>

Surface expression of CALR does not always occur after administration of cytotoxic therapies, as certain chemotherapies such as cisplatin or melphalan are unable to induce this feature of ICD.<sup>148</sup> Moreover, there are tumor-specific differences in surface exposure of CALR, as prototypical ICD inducers such as doxorubicin do not expose CALR on B-cell lymphoma<sup>148</sup> or acute myeloid leukemia cells.<sup>149</sup> Despite this, a retrospective analysis of non-small cell lung cancer patients showed that CALR expression on tumor cells was significantly correlated with eIF2 $\alpha$  phosphorylation and disease outcome.<sup>131</sup> Higher CALR expression was associated with tumor infiltration by DCs and effector memory T-cell subsets that conferred prolonged survival.<sup>131</sup> Tumor-intrinsic determinants of CALR surface exposure are currently lacking. Recently, the promoter of the tumor suppressor retinoblastoma 1 (RB1) has been shown to dually control the expression of 7.1 kb non-coding RNA located upstream of the RB1 gene (ncRNA-RB1).<sup>137</sup> The study identified that CALR is a novel target of ncRNA-RB1. Depletion of ncRNA-RB1 contributes to a failed surface exposure of CALR during mitoxantrone treatment resulting in reduced phagocytosis of dying cells.<sup>137</sup> While surface CALR on dying cells promotes their phagocytic uptake, this process is often counteracted by tumors through overexpression of the anti-phagocytic signal CD47.<sup>151</sup> CD47 is a pentaspanin cell surface protein that counters phagocytosis through ligation of its signal regulatory protein a (SIRP $\alpha$ ) receptor on phagocytic cells.<sup>150</sup> Recently, oncogenic MYC was found to subvert immunosurveillance by upregulating the expression of CD47 which resulted in poor macrophage infiltration in multiple tumor types, including lymphoma/leukemia and liver cancer<sup>138</sup> (Figure 4c).

### Extracellular ATP

Extracellular ATP actively secreted or passively released from dying tumors is one of the immunomodulatory molecules facilitating immune-mediated detection of tumors during ICD. The mechanism of ATP release during ICD depends on the type of lethal stimulus. Anthracyclines induce ATP release via caspase-dependent activation of pannexin 1 channels,

lysosomal exocytosis, and plasma membrane blebbing.<sup>151</sup> Moreover, autophagy is indispensable for the release of ATP during anthracycline-induced tumor cell death. However, ATP secretion after hypericin-based PDT operates independently of autophagy and involves PERK-regulated proximal secretory pathways, as well as PI3K-dependent exocytosis. ATP exerts its immunomodulatory effect via activation of purinergic P<sub>2</sub>X<sub>7</sub> and P<sub>2</sub>Y<sub>2</sub> receptors (P<sub>2</sub>RX<sub>7</sub>, P<sub>2</sub>RY<sub>2</sub>) on myeloid cells. In mouse DCs, extracellular ATP binds to P<sub>2</sub>RX<sub>7</sub> to initiate inflammatory signaling and the release of IL-1 $\beta$  to activate IL17-producing  $\gamma\delta$  T cells.<sup>152</sup> Thus, the lack of inflammasome signaling components and/or IL17 signaling aborts the immunogenic effects of anthracycline-induced cell death.<sup>153</sup>

The excessive inflammatory effects of extracellular ATP are countered by a negative feedback mechanism to limit tissue injury.<sup>154</sup> Precisely, a system consisting of the ecto-enzymes CD39 and CD73, together with receptors of the adenosinergic pathway, converts immunostimulatory ATP into immunosuppressive adenosine.<sup>155</sup> ATP in the extracellular environment is broken down to adenosine monophosphate (AMP) by ecto-ATPases such as CD39.<sup>155</sup> The ecto-5'-nucleotidase CD73 breaks down AMP into adenosine. CD73-derived adenosine in the extracellular environment exerts its immunosuppressive effects by binding to one of the four G-protein-coupled adenosine receptors.<sup>155</sup> CD73 transcription is directly regulated by HIF-1 $\alpha$ ,<sup>156</sup> explaining why the hypoxic tumor microenvironment is often associated with high expression of CD73 in cancer cells, endothelial cells, fibroblasts, lymphocytes and myeloid cells. Tumor-derived CD73 can potently inhibit anti-tumor immunity by suppressing T and NK cell function.<sup>157,158</sup> High amounts of CD73 in triple-negative breast cancer<sup>132</sup> and high-grade serous ovarian cancer<sup>159</sup> patients are negatively associated with tumor infiltration and overall patient survival. Patients with high levels of CD73 and low levels of tumor-infiltrating leucocytes show poor clinical outcome.<sup>132</sup> Oncogenic pathways such as Wnt and downstream  $\beta$ -catenin,<sup>160</sup> MAPK,<sup>161</sup> EGFR<sup>162</sup> and AKT signaling promote CD73 expression on tumor cells<sup>163</sup> (Figure 4c). Moreover, increased CD73 was significantly associated with TP53 mutations in melanoma patients.<sup>164</sup> BRAF-targeted therapy with either dabrafenib or vemurafenib showed reduced levels of CD73 in biopsies of melanoma patients.<sup>164</sup>

## HMGB1

HMGB1 is a highly conserved nonhistone chromatin-binding protein abundantly expressed in all eukaryotic cells.<sup>165,166</sup> HMGB1 can be released passively or actively,<sup>167,168</sup> although the molecular mechanisms that initiate the release of HMGB1 from tumor cells undergoing ICD remain to be elucidated. Extracellular HMGB1 has several immunomodulatory properties<sup>166,169</sup> based on its redox state and post-translational modifications.<sup>170</sup> The immunogenicity of anthracycline-treated dying apoptotic cells depends on the passive release of HMGB1 that binds to TLR4 in innate immune cells. Extracellular HMGB1 mediates its effects by binding to PRRs such as TLR4<sup>171</sup> and RAGE.<sup>172</sup> Various types of ICD inducers such as anthracyclines,<sup>173</sup> radiation<sup>174–176</sup> and oncolytic

viruses<sup>5</sup> stimulate the secretion of HMGB1. The immunotherapeutic effects of anthracycline therapy failed in TLR4<sup>-/-</sup> and Myd88<sup>-/-</sup> mice suggesting that the HMGB1-TLR4-MYD88 axis is essential to stimulate the maturation of dendritic cells thereby enhancing their ability to cross-present tumor antigens.<sup>173</sup> In human breast cancer patients, a polymorphism in TLR4 that affects the binding of HMGB1 predicts relapse after anthracycline therapy.<sup>173</sup> In summary, the role of HMGB1 in anticancer immunity is complex and future studies should aim to elucidate tumor-specific and microenvironment-dependent immune effects of extracellular HMGB1.

## Conclusions and future perspectives

The current state-of-the-art suggests that cancer cell-intrinsic factors can affect the activation and subversion of cancer cell ICD, although detailed mechanistic studies elucidating specific biological interactions are lacking. Understanding the intimate relationship between oncogenic factors and ICD will yield essential biological information on which oncogenic factors to target depending on the malignant indication. These investigations will be useful for the selection of patients that would benefit from immunomodulation upon ICD-inducing treatments. This knowledge will help maximizing the potential of ICD-inducing therapeutics for cancer patients and provide rationale for personalized medicine based on the genetic profile of tumors. We expect that the combination of targeted therapies against tumor-intrinsic oncogenic pathways with ICD-inducing agents will further stimulate the cancer-immunity cycle, and particularly the cytotoxic T cell response. This basic research will contribute to clinical oncology through the development of novel biotherapeutics.

## Disclosure statement

JGP is named as an inventor on patents for cancer vaccination involving an oncolytic rhabdovirus. These patents have been licensed to Turnstone Biologics of which JGP is a shareholder. GK is a cofounder of Samsara Therapeutics, everImmune, and Therafast Bio. STW has no relevant conflict of interest to declare.

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