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"Επαγωγή Ανοσογόνου κυτταρικού θανάτου κατά την αντικαρκινική θεραπεία - Κλινικές εφαρμογές"

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CONTENTS

ΠΕΡΙΛΗΨΗ. Η αποτελεσματική θανάτωση των καρκινικών κυττάρων είναι ο κύριος στόχος της αντικαρκινικής θεραπείας. Η αποφυγή του κυτταρικού θανάτου είναι ένα από τα χαρακτηριστικά των καρκινικών κυττάρων που συχνά εμφανίζουν αντίσταση στη θεραπεία. Ως εκ τούτου, η διαλεύκανση του μηχανισμού του κυτταρικού θανάτου που επάγεται κατά τη διάρκεια της αντικαρκινικής θεραπείας είναι θέμα αιχμής εδώ και μια δεκαετία περίπου διότι καθορίζει σε σημαντικό βαθμό την αποτελεσματικότητα της αντικαρκινικών παραγόντων. Στο σχεδιασμό αποτελεσματικών αντι-καρκινικών ανοσοθεραπειών που οδηγούν σε αναίρεση του φαινομένου της αντίστασης των καρκινικών κυττάρων στην αντικαρκινική ανοσία, ζητούμενο είναι η αξιοποίηση του ανοσογόνου δυναμικού των καρκινικών κυττάρων που πεθαίνουν ή είναι ήδη νεκρά Συχνά, ο κυτταρικός θάνατος που παρατηρείται σε αυτές τις συνθήκες συνδέεται με την ενεργοποίηση μονοπατιών που σηματοδοτούν κίνδυνο (danger signaling pathways) και την απελευθέρωση μοριακών προτύπων που σχετίζονται με βλάβες (DAMPs). Ο μηχανισμός αυτός που ονομάζεται Ανοσογόνος Κυτταρικός Θάνατος (ICD: Immumogenic Cell Death) αυξάνει σημαντικά την ανοσογονικότητα των καρκινικών κυττάρων που πεθαίνουν, προκύπτει μέσω προγραμματισμένου κυτταρικού θανάτου (Απόπτωση και/ή Νεκρόπτωση) ενώ επάγεται *in vitro* και *in vivo* από πολλούς χημειοθεραπευτικούς παράγοντες. Στο πλαίσιο της ανασκόπησης θα παρουσιαστούν οι πρόσφατες εξελίξεις στο τομέα, θα σχολιαστούν οι προκλήσεις που πρέπει να απαντηθούν στο άμεσο μέλλον, με έμφαση στη διασύνδεσή του ICD με την ανοσοθεραπεία και την αξιοποίησή του στη συνδυαστική θεραπεία έναντι του καρκίνου στην κλινική πράξη.

"Induction of immunogenic cell death during anticancer therapy-Clinical applications and challenges"

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Abstract. Effective killing of cancer cells is the main goal of cancer treatment. Avoiding cell death is one of the characteristics of cancer cells that often show resistance. Therefore, the investigation of the mechanisms of cell death induced during anticancer treatment has been a cutting-edge issue for about a decade because it significantly determines the effectiveness of anticancer agents. In the design of effective anti-cancer immunotherapies that lead to the undoing of the phenomenon of anti-cancer immunity, it is necessary to exploit the immunogenic potential of cancer cells that die or are already dead. The cellular death observed under these conditions is often associated with the activation of risk signaling pathways and the release of molecular patterns related to lesions (Damage-associated molecular patterns: DAMPs). This mechanism called Immunogenic Cell Death (ICD) significantly increases the immunogenicity of dying cancer cells, occurs through programmed cell death (Apoptosis and/or Necroptosis) and is induced *in vitro* and *in vivo* by many chemotherapeutic agents. This work will present recent developments in the field and comment on the challenges that need to be met in the near future, with an emphasis on linking ICD with immunotherapy and its use in combined cancer treatments in the clinical practice.

1. Introduction

As Mayo Clinic reports, cancer refers to any one of a large number of diseases characterized by the development of abnormal cells that divide uncontrollably and have the ability to infiltrate and destroy normal body tissues. Cancer also has the ability to metastasize, i.e., to spread throughout the body (https://www.mayoclinic.org/diseases-conditions/cancer/). Cancer is the second-leading cause of death in the world and places a heavy burden on health services and society. However, survival rates are improving for many types of cancer, due to improvements in cancer screening and treatment. Human tumor pathogenesis involves multistep processes that occur rationally and could enable normal cells to become tumorigenic and ultimately malignant. According to Hanahan and Weinberg the six hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing

angiogenesis, and activating invasion and metastasis[1]. Herein, we will focus on the ways cancer cells die and the mechanisms underlying their effort to evade cell death induced by anti-cancer treatment in order to acquire resistance.

The balance between cell death and survival is fundamental for eukaryotic cell development and tissue homeostasis. There are several ways through which normal cells die or survive. When they lose the ability of balancing, tissues cannot escape from multifactorial diseases, such as cancer. There are several pathways of death, characterized by different biochemical, molecular and mechanistic steps. On the other hand, eukaryotic cells can die by accident, suicide, or murder. Accidental cell death, usually as a result of severe stress, is not under the control of specific genes or gene products, whereas regulated cell death, suicide or murder, is genetically-mediated and tightly controlled[2,3]. The decision between cell survival and death following DNA damage rests on factors involved in DNA damage recognition, DNA repair and damage tolerance as well as the activation of cell death mechanisms. The pathways dictating cell fate are entwined and have key roles in cancer initiation and progression[4]. All the different modalities of regulated cell death maintain the same purpose. They respond to microenvironmental perturbations to promote cellular and organismal homeostasis in both physiological and pathological conditions, providing obvious advantages to multicellular organisms[5,6].

Cell death processes have been defined on the basis of their specific morphological features (e.g., apoptotic, autophagic, or necrotic), their metabolic and biochemical characteristics (e.g., loss of mitochondrial transmembrane potential, exposure of phosphatidylserine (PS) on the outer leaflet side, or rupture of plasma membrane integrity), their enzymatic and catabolic activities (involving or not caspases, receptor-interacting protein kinases (RIPKs), mixed lineage kinase domain-like proteins (MLKLs), or cathepsins), and in relation to their ability to elicit an inflammatory reaction or stimulate an immune response (Immunogenic cell death, ICD). Considering the above criteria, cell death has conventionally been classified into three distinct types: apoptosis (Type I), autophagy or autophagic cell death (Type II), and necrosis (Type III)[7]. All these processes, executed in a cell-autonomous manner, can be induced in the targeted stressed cells or at a distance, in the neighboring cells (through bystander effects) and are known as cell-autonomous death (CAD). Despite major progresses that have been made in the field, the relative contribution of both direct and bystander-signal-mediated killing triggered by typical CAD remains poorly explored[8]. The examination of additional unconventional cell death modes (such as entosis or emperitosis) has revealed the existence of cell death processes that are elicited after the engulfment of live cells by neighboring live cells, known as non-cell-autonomous death (NCAD)[8]. In general, during the last decades, unconventional forms of cell death, namely necroptosis, pyroptosis, phagoptosis/ entosis and ferroptosis, have been extensively studied. The common molecular characteristic of these novel cell death modes is that they are highly regulated. Regulated cell death (RCD) is further categorized into two groups: caspase-dependent (e.g., apoptosis and pyroptosis) and caspase-independent RCD (e.g. necroptosis, ferroptosis, parthanatos)[9](Figure 1).

Caspases are a family of cysteine proteases. The function of these enzymes appeared to be associated not only with apoptosis but also with inflammation. Recent discoveries, however, have unveiled their roles in mediating and suppressing two regulated forms of necrotic cell death, termed pyroptosis and necroptosis[10]. Their function is not limited to

cell death. Non-apoptotic roles of caspases include proliferation, tumor suppression, differentiation, neural development as well as axon guidance and aging[11]. The human caspase family is divided into three main groups, primarily based on sequence similarities and biological functions. Group I comprises the inflammatory caspases-1, -4, and -5 (caspase-11 in mouse), based on the commonalities of having a long caspase-recruitment domain and a preference for a large aromatic or hydrophobic residue at position P4. Within this group, caspase-1 is the best characterized and well known for processing IL-1β involved in inflammation. Group II comprises the apoptotic effector caspases-3, -6, and -7 that share a similar short pro-domain, and are usually described as the 'executors of apoptosis'. Finally, group III includes the human initiator caspases-8, -9, and -10, all of which contain a long pro-domain and prefer substrates with a leucine or valine at position P4. These broad group classifications are admittedly imperfect. For example, although caspase-2 has been characterized as an initiator caspase (group III) because of its long pro-domain, its substrate specificity is more similar to 'executioner-like' group II caspases. Conversely, caspase-6 has been characterized as an executioner caspase because of its short pro-domain and sequence recognition motif, but caspase-6 activation alone is not sufficient to lead all cells to apoptosis[12]. Based on their function, caspases can be divided into inflammatory caspases (caspase-1, -4, -5, -11 and -12) and apoptotic caspases which initiate and execute an immunologically silent form of programmed cell death known as apoptosis. Members of the apoptotic caspase family include the initiator caspases, caspases-2, -8, -9 and -10, and the effector caspases, caspases-3, -6 and -7[13]. Caspases are first synthesized in cells as zymogens and their activation requires either an allosteric conformational change, specific cleavage after a selective aspartate residue, or both, to lead to the formation of tetrameric active enzymes[10]. Proteolytic cleavage leads to changes in cell morphology such as membrane blebbing, DNA fragmentation, phosphatidylserine exposure at the cell surface, and formation of apoptotic vesicles. Caspases are expressed by both immune and non-immune cells and in many tissues and organs.

2. Main types of cell death

2.1. Apoptosis

The molecular mechanisms regulating apoptosis have been extensively investigated in multiple organisms over the last 30 years. It is now established that apoptosis can proceed following the extrinsic and/or the intrinsic mitochondrial pathway.

Extrinsic apoptosis is mediated by membrane receptors, especially by death receptors such as FAS cell surface death receptor, also known as CD95 and TNF receptor superfamily member 1A (TNFRSF1A), also known as TNFR1, and is driven by initiator caspases -8 and -10[9]. This pathway involves the formation of death-inducing signaling complexes (DISC) comprising FAS-associated death domain protein (FADD) and/or TNFR-associated death domain protein and activation of caspase-8, which directly activates effector caspases. Caspase-8 also cleaves BH3 interacting domain death agonist (Bid) to a truncated form (tBid), which engages the mitochondrial pathway to amplify the apoptotic

response. Bid is a pro-apoptotic protein of the B-cel lymphoma 2 (Bcl-2) family that is crucial for death receptor-mediated apoptosis in many cell systems[14]. In general, DR-induced cell death is critical for immune system function and homeostasis.
Intrinsic apoptosis is initiated by cytotoxic drugs or DNA damage. This pathway involves activation of p53 and pro-apoptotic proteins Bcl-2 associated X (BAX) and Bcl-2 homologous antagonist (BAK) which induce mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release. Apoptotic protease-activating factor 1 (Apaf-1) associates with cytochrome *c* into a large multimeric complex called the apoptosome to activate caspase-9. Then caspase-9 is cleaved and activates other effector caspases such as caspase-3.
Once initiator caspases are activated through the extrinsic or intrinsic apoptosis pathways, they mediate activation of effector caspases-3, -6 and -7. Apoptosis is characterized by typical morphological and biochemical hallmarks, including cell shrinkage, nuclear DNA fragmentation and membrane blebbing[15].

2.2. Autophagy

Autophagy is a catabolic process whose activation may help cancer cells adapt to cellular stress, such as nutrient deprivation, hypoxia, growth factor withdrawal, and infections. Thus, today's consensus is that autophagy is a response to stress or damage[6]. In this process, parts of the cytosol and specific organelles are engulfed by a double-membrane structure, known as the autophagosome, and eventually degraded. Through degradation and recycling of cellular components, autophagy supplies a continual source of metabolic building blocks to overcome the cellular deficiency. Autophagy-related genes (*Atg*) control the process of autophagy. The products of these *Atg* genes are regulated by nutrient (mammalian target of rapapmycin [mTOR]), energy (AMP-activated protein [AMPK]) and stress (hypoxia-inducible factors [HIFs]) sensing mechanisms that turn the pathway on and off within the cell[16]. Once activated, a series of ATG protein complexes orchestrate the formation of autophagosomes that capture cytoplasmic cargo such as damaged proteins, organelles, lipids, and glycogen. Lysosomes, organelles that contain hydrolytic enzymes, help achieve this goal through their fusion with autophagosomes.
In the past, researchers believed that autophagy is only a survival mechanism; nevertheless, there are a few examples of autophagic cell death in which components of the autophagic signaling pathway actively promote cell death[3]. Of note, although autophagy (i.e., the membrane engulfment and catabolic degradation of parts of the cytoplasm) is a well-defined process, its function as an active cell death mechanism remains highly controversial and the term “autophagic cell death” (ACD) is currently under intense debate.

2.3. Necrosis

Necrosis can occur when tissues or cells are exposed to external damage such as infections, toxins or trauma[15]. In such cases, cell death is passive and does not require the activation of any particular signaling pathway as observed in apoptosis. This unregulated digestion leads to loss of membrane integrity and swelling of subcellular organelles (oncosis). A typical inflammatory response follows with the activation of inflammasomes (cytosolic

multiprotein oligomers) of the innate immune system. It promotes proteolytic cleavage, maturation and secretion of the pro-inflammatory cytokines interleukin 1β (IL-1β) and interleukin (IL-18). Necrosis has long been considered as an uncontrolled type of cell death. However, necrotic cell death is not always an accidental or passive process and can also be the result of a directed signaling cascade. In the late 1980s, it became clear that necrosis can also function as an alternative programmed mode of cell death, triggered by the same death signals that induce apoptosis[17]. Note that the rupture of plasma membrane, a hallmark of necrotic morphology, can also be observed at late stages of an apoptotic or autophagic cell death program, when dead cells fail to be cleared by phagocytosis. This process is referred to as secondary necrosis and is independent of any other signaling event initially engaged (apoptotic or autophagic). The best-characterized form of programmed necrosis is RIP-kinase-dependent (RIPK) necrosis also referred to as "necroptosis" [18].

2.4. Necroptosis

Necroptosis represents the best studied form of programmed necrosis and seems to be a cellular response to environmental stress that can be caused by chemical and/or mechanical injury, inflammation, or infection. In the same way that caspases are key intracellular mediators of apoptosis, receptor-interacting protein kinases (RIPKs) are essential mediators of necroptosis. The kinase activity of RIPK3 is essential for necroptosis but also dictates whether a cell activates caspase- 8 and dies through apoptosis. The current understanding of necroptosis has largely developed around the TNF-α receptor system. TNF-α is a pleiotropic molecule capable of inciting a survival, apoptotic or necroptotic response based on the assembly of sequential but mutually exclusive cell death complexes. Depending on the cellular context, engagement of TNF-α can trigger the formation of complex I, a prosurvival complex that signals through NF-κB. However, in conditions under which RIPK1 is de-ubiquitinated, the complex becomes an apoptotic complex IIa. Furthermore, the absence of caspase-8, in addition to elevated levels of RIPK3, alters the complex to IIb, also called the necrosome. This necrosome contains RIPK1, RIPK3, and Fas-associated protein with death domain that allow the cell to undergo necroptosis via direct phosphorylation of mixed-lineage kinase domain-like protein (MLKL) by RIPK3. Phosphorylation of MLKL results in a pore-forming oligomer that punctures the plasma membrane and causes subsequent cell death with distinct immunologic consequences[18]. Although apoptosis and necroptosis frequently have common triggers, the intracellular signaling pathways leading to the execution of apoptosis and necroptosis differ. The possibility that tumor cells resistant to death receptor-induced apoptosis could shift their mode of cell death toward necroptosis could have an impact on immunogenicity and the subsequent action of immune surveillance mechanisms as well as on the efficacy and side effects of immunotherapy treatments[19].

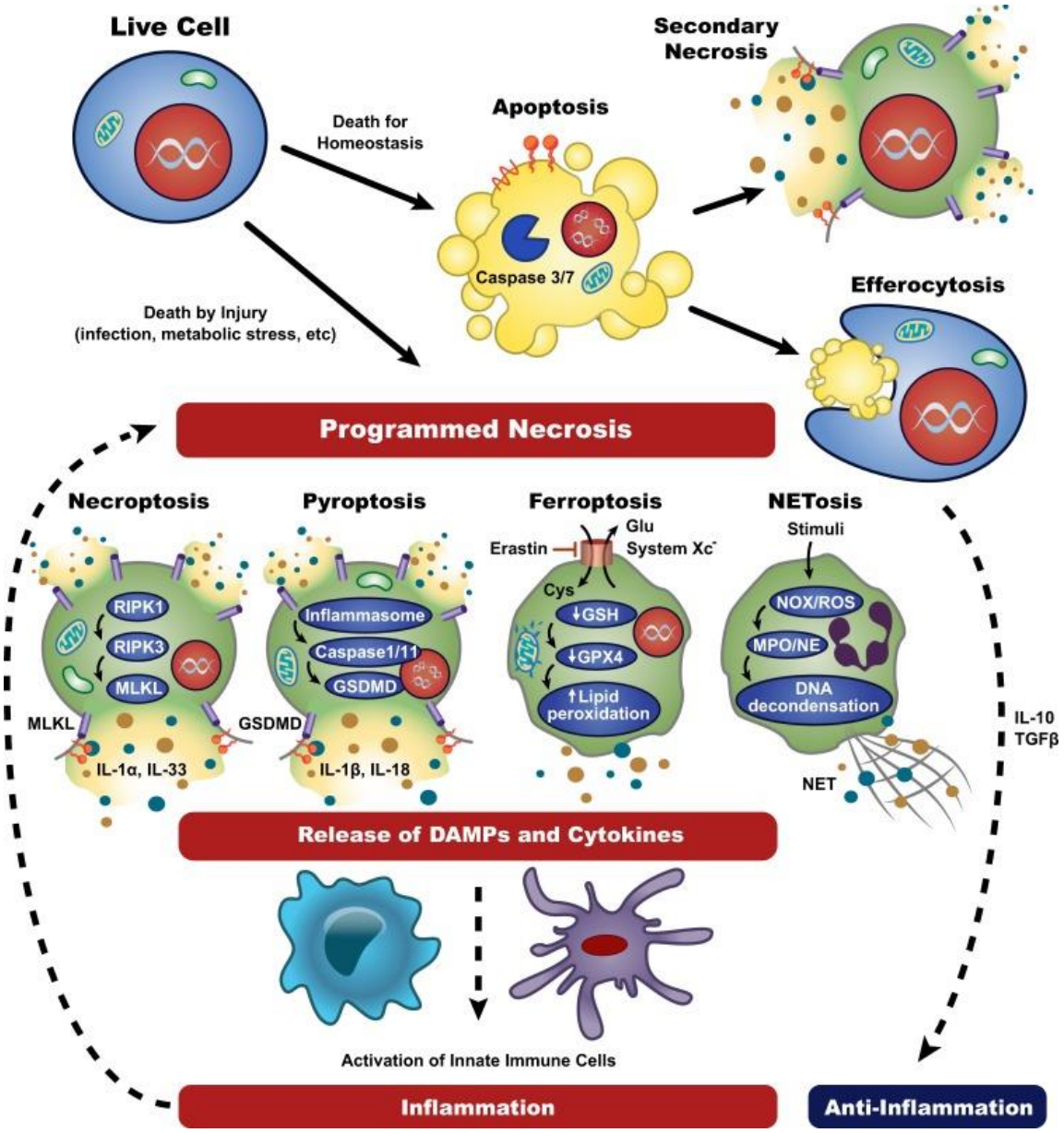

Figure 1. Main types of cell death and their molecular pathways. Depending on death stimuli and context, live cells can undergo apoptosis or programmed necrosis. When caspase 3/7-dependent apoptotic cells are timely scavenged by efferocytosis, macrophages release anti-inflammatory cytokines and prevent unwanted inflammation. In the absence of effective clearance, apoptotic cells are proceeded to secondary necrosis and elicit some inflammatory responses. Upon damage signals such as infection or metabolic stress, cells trigger genetically programmed necrosis. Necroptosis and pyroptosis are RIPK3-MLKL- and inflammasome-GSDMD-mediated processes, respectively. In contrast, ferroptosis is triggered by lipid peroxidation, and shows damaged mitochondria and reduced cellular volume. NETosis is a ROS-induced lytic cell death resulting in the extrusion of neutrophil extracellular traps (NETs), consisting of genomic DNA complexed with cellular proteins. Programmed necrotic cells generally release DAMPs and inflammatory cytokines that stimulate innate immune cells and promote necroinflammation

Source: Ho Kim E. , Wong S. , Martinez J. Programmed Necrosis and Disease:We interrupt your regular programming to bring you necroinflammation.Cell Death Differ. 2019 Jan;26(1):25-40.

2.5. Pyroptosis

This pro-inflammatory caspase-1-dependent process, initially described in 1992 and observed in rapidly dying Salmonella-infected macrophages by Cookson and Brennan in 2001, is definitely different from non-inflammatory apoptosis[20]. “Pyro” describes fire or fever and “potosis”/”ptosis” signifies a pro-inflammatory programmed cell death. Recent studies have shown that pyroptosis is also triggered by caspase-4 and -5[19]. In the canonical model of pyroptosis inflammasome proteins such as NLR family pyrin domain containing 3 (NLRP3), recognize cellular stressors including bacteria, viruses, toxins, chemotherapeutic drugs and damage-associated molecular patterns (DAMPs). The oligomerization of NLRP3 activates Caspase-1. Thus, playing the main role in this pathway of cell death, caspase-1 activates the inflammatory cytokines interleukin 1β and interleukin 18. However, caspase-1 activation can result not only in the production of activated inflammatory cytokines, but also rapid cell death characterized by plasma-membrane rupture and release of pro-inflammatory intracellular contents. Caspase-1 cleaves specific members of the pore-forming gasdermin gene family such as gasdermin D (GSDMD) to release membrane pore-forming GSDMD-N domain (activated region) from an inhibitory C-terminal fragment. GSDMD-N pores promote the release of activated cytokines and DAMPs. Caspase 1-dependent cell death is a programmed process of cellular self-destruction mediated by caspases, and therefore was not initially distinguished from apoptosis[21]**.** Pyroptosis, like necroptosis, is a lytic cell death modality allowing the release of potential immunostimulatory molecules.

2.6. Phagoptosis

Phagoptosis is a recently recognized form of cell murder that occurs when a phagocyte consumes an otherwise viable cell. This process is distinct from phagocytosis of apoptotic or necrotic cells[3] . Phagocytes eat cells that: i) expose 'eat-me' signals, ii) lose 'don’t-eat-me' signals, and/or iii) bind opsonins. Live cells may express such signals as a result of cell stress, damage, activation or senescence, which can lead to phagoptosis. For example, phosphatidylserine is an "eat-me" signal that, when exposed on the surface of a cell, triggers phagocytes to eat up the cell. Phosphatidylserine is normally found in the interior of healthy cells, but can become exposed on the surface of dying, activated or stressed cells. Phagocytosis of such cells requires specific receptors on the phagocyte that recognize either phosphatidylserine directly or opsonins bound to the phosphatidylserine or other "eat-me" signals, such as calreticulin. "Don't-eat-me" signals include the immunoglobin Cluster of Differentiation 47 (CD47), which when expressed on the surface of a cell, inhibits its phagocytosis by activating the signal-regulatory protein alpha (SIRPα) receptors on the phagocyte. Opsonins are normally soluble proteins, which when bound to the surface of a cell induce phagocytes to phagocytose that cell. Phagoptosis may be the

most abundant form of cell death physiologically as it mediates the turnover of erythrocytes, neutrophils and other cells [22,23]. More recently it has become clear that most human cancer cells overexpress CD47 on their surface to protect themselves from being phagocytosed, and if this 'don't-eat-me' signaling is blocked then a variety of cancers can be cleared from the body. Thus it would appear that phagoptosis is an important host defense mechanism against cancer, which tumour cells can effectively suppress. Reversing this tumor-induced suppression is therefore an attractive therapeutic option.

2.7. Ferroptosis

Ferroptosis is an iron-dependent and reactive oxygen species *(*ROS)-reliant type of cell death. The morphological and biochemical characteristics involve cell volume shrinkage and increased mitochondrial membrane density[24]. In addition, the presence of iron, particularly divalent iron, greatly accelerates lipid peroxidation of saturated fatty acids in humans. During iron-involving oxidative phosphorylation in mitochondria, cells produce reactive oxygen species (ROS) along with the generation of ATP. ROS levels that exceed the cell's anti-oxidation capacity can lead to an oxidative stress response, which directly and indirectly damages large molecular substances such as proteins, nucleic acids and lipids, leading to cell injury or death. Ferroptosis differs from apoptosis and necrosis in the traditional sense and results from the accumulation of iron-dependent lipid peroxide[25]. The fast-growing studies of ferroptosis in cancer have boosted a perspective for its usage in cancer therapeutics [24,26].

3. Cell Death and Immunosurveillance in anticancer treatment

Over the past five decades, remarkable achievements have been made in the fight against cancer, starting from understanding cancer mechanisms to actual patient treatment. Scientific discoveries and technological advances, including modern molecular biology methods, high-throughput screening, structure-based drug design, combinatorial and parallel chemistry, and sequencing of the human genome led to the discovery of novel, more effective drugs. However, the increasing cost of drug development and decreasing number of truly efficient medicines approved by the US Food and Drug Administration (FDA) present unprecedented challenges for the pharmaceutical industry and patient healthcare, including the field of oncology.

A limited number of cancers can be completely cured using "traditional" treatment approaches such as chemotherapy and radiotherapy. However, the success of cancer treatments varies enormously depending on the specific type of cancer diagnosed and the stage of the disease at diagnosis. Very often, resistance occurs against every effective anticancer drug, developed through numerous mechanisms and consequently, many patients become refractory to treatment. A variety of factors contribute to multi-drug resistance (MDR), including different mechanisms depending on the structure and action of the drug administered, cell death inhibition and suppression of apoptosis, alterations in drug metabolism, epigenetic modulation, enhancement of DNA repair mechanisms, and gene amplification. The development of MDR to chemotherapy remains a major challenge

in treating cancer[27]. Among these mechanisms, the response or adaptation of cancer cells to anticancer drug-induced tumor microenvironmental stress is a vital cause of chemotherapy resistance[28].

One of the most important advances in cancer research in recent years is the recognition that current cancer therapies, for example, chemotherapy, γ-irradiation, immunotherapy or suicide gene therapy, primarily exert their antitumor effect by triggering cell death mostly by apoptosis[111]. Defective apoptosis represents a major causative factor in the development and progression of cancer. The ability of tumor cells to evade apoptosis and/or other cell death modes can play a significant role in their resistance to conventional therapeutic regimens. Furthermore, autophagy-mediated cell death mechanisms contribute to the efficacy of anticancer drugs[28]. Although the pro-survival or anticancer effect of autophagy is still controversial, *in vitro* and *in vivo* experimental evidence suggests that autophagy rather facilitates cancer cell resistance to chemotherapy, and inhibition of autophagy may potentiate resensitization of drug-resistant cancer cells to the anticancer therapy[28]. Thus, understanding the novel function of autophagy may allow the development of a promising therapeutic strategy to enhance the effects of chemotherapy and improve clinical outcomes in the treatment of cancer patients.

On the other hand, the development of immunotherapies against cancer is also a major breakthrough in oncology. Immunotherapies use the body's own immune system to find and destroy cancer cells providing long-term clinical benefit and prolonged survival. The concept of "immunotherapy" refers to "antitumor immunity", of both innate and adaptive immune responses, which lead to tumor control. This process is based on "immunosurveillance", the ability of cells of the immune system to look for and identify cancerous cells in the body. Cytotoxic lymphocytes (CL), cytotoxic T (CD8+ Tc) and natural killer (NK) cells, are the main effector cells during cancer immunosurveillance[29]. Other cell types, such as macrophages, mast cells, or dendritic cells, may also kill transformed cells. Although triggered via distinct receptors, Tc and NK cells use the same basic mechanisms to destroy their target cells[30]. Both effector pathways trigger programmed intracellular events in target cells, leading in the majority of cases to apoptotic cell death.

In fact, cancer immunosurveillance, which is driven largely by activated effector T cells, is impaired at different levels by several obstacles imposed by the increasingly hostile tumor microenvironment (TME). T-cell activation relies on the interaction of the T-cell receptor with antigens presented as peptides through the major histocompatibility complex (MHC) by professional antigen-presenting cells (APCs) named dendritic cells (DCs). Thereafter, activated CD8+ T cells can recognize Tumor Associated Antigens (TAAs) presented through a MHC class I molecule on cancer cells and induce their killing via the perforin-granzyme and/or Fas ligand (FasL)/tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) systems. However, this depends on the degree of functional inhibition by the TME and the presence of immunosuppressive regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs). In this scenario, blood and lymphatic vasculature have important roles as physical and functional barriers for tumor-infiltrating immune cells and TAA/TAA-presenting DC drainage to the lymph nodes, respectively[31]. It is worth noting that CD4+ regulatory T cells (Tregs) are highly immune suppressive and physiologically

play a central role in maintenance of self-tolerance and immune homeostasis; yet in malignant tumors they promote tumor progression by suppressing effective antitumor immunity[32]. While the recognition of peptide–MHC by the T-cell receptors plays a central role in the process of T-cell-mediated immunity, additional cell surface co-receptors are mandatory for the modulation of the immune response, either positively or negatively. Two of these inhibitory co-receptors, called immune checkpoints, are involved in adaptive immune resistance and T-cell tolerance and have been exploited clinically with the development of checkpoint-blocking monoclonal antibodies. The two receptors comprise the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152) and the programmed cell death receptor 1 (PD-1 or CD279) and its ligand (PD-L1, also named CD274 or B7-H1)[33].

Anti-cancer drugs can be broadly classified into two basic categories: cytotoxic and targeted agents based on their mechanism of action. Cytotoxic agents can kill rapidly dividing cells by targeting components of the mitotic and/or DNA replication pathways. Targeted agents block the growth and spread of cancer through interacting with molecular targets that are involved in the pathways relevant to cancer growth, progression, and spread [34]. These drugs are designed to attack cancer cells while causing less damage to normal cells. It is currently accepted that cancer cells may die in response to anti-cancer therapies through regulated cell death programs, which may either repress or increase their immunogenic potential. In particular, the induction of Immunogenic Cell Death (ICD) in cancer cells, which is hallmarked by the emission of damage-associated molecular patterns (DAMPs), molecules analogous to pathogen-associated molecular patterns (PAMPs) found on micobes, acting as danger signals/alarmins, is of great relevance in cancer therapy[35]. This emerging combination of DAMPs, immunogenic cell death and anticancer therapeutics may be the key towards the elimination of cancer-related mortalities, in the near future[36]. The characteristics of an immune response to cell death (e.g., immunogenic vs. tolerogenic responses) are determined by the precise molecular signaling between dying cells and local immune cells[37]. Immunogenic cell death (ICD) refers to all the forms of accidental and regulated cell death that stimulate a T cell-dependent immune response following the release of dead cell-derived antigens. Both accidental cell death and regulated cell death can stimulate the immune response. Dying cells activate adaptive responses associated with the expression and secretion of DAMPs in the microenvironment, i.e., molecules that are not accessible by the immune system under physiological conditions[38] . (Figure 2)

Interestingly, DAMPs are molecules that have a physiological role inside the cell, but acquire additional functions when they are exposed to the extracellular environment: they alert the body about danger, stimulate an inflammatory response, and finally promote the regeneration process[39]. DAMPs such as the endoplasmatic reticulum (ER) chaperone named calreticulin (CRT), secreted ATP, passively released non-histone chromatin-binding protein high mobility group box 1 (HMGB1) are vital for the secretion of immunostimulatory cytokines, such as type I interferons [40] and the induction of immunogenic cell death (ICD) in cancer cells[36]. When emitted in the correct spatiotemporal pattern, such DAMPs recruit antigen-presenting cells, including dendritic cells (DCs), to the site of ICD and activate them to engulf dead cancer cell-associated

antigens, process and present them to CD4+ and CD8+ T lymphocytes in the presence of the appropriate co-stimulatory signals, resulting in the priming of a robust tumor antigen-specific immune response[41]. More specifically, DAMPs released from cancer cells ligate receptors and surface molecules on DCs and promote the differentiation of immature DCs to a mature phenotype. DCs recognize 'eat me' signals when calreticulin is exposed to the cell surface triggered by ER stress and the production of reactive oxygen species (ROS)[42]. Similarly, ATP released to the tumor microenvironment (TME) is regarded as a 'find me' signal, which triggers P2X purinoceptor 7 (P2X7) receptors on DCs and is responsible for the activation of NALP3-ASC-inflammasome and the secretion of IL-1β. However, while the signaling pathways governing surface exposure of CRT have been delineated to some extent, insufficient information exists on the molecular pathway behind ATP secretion[43] .

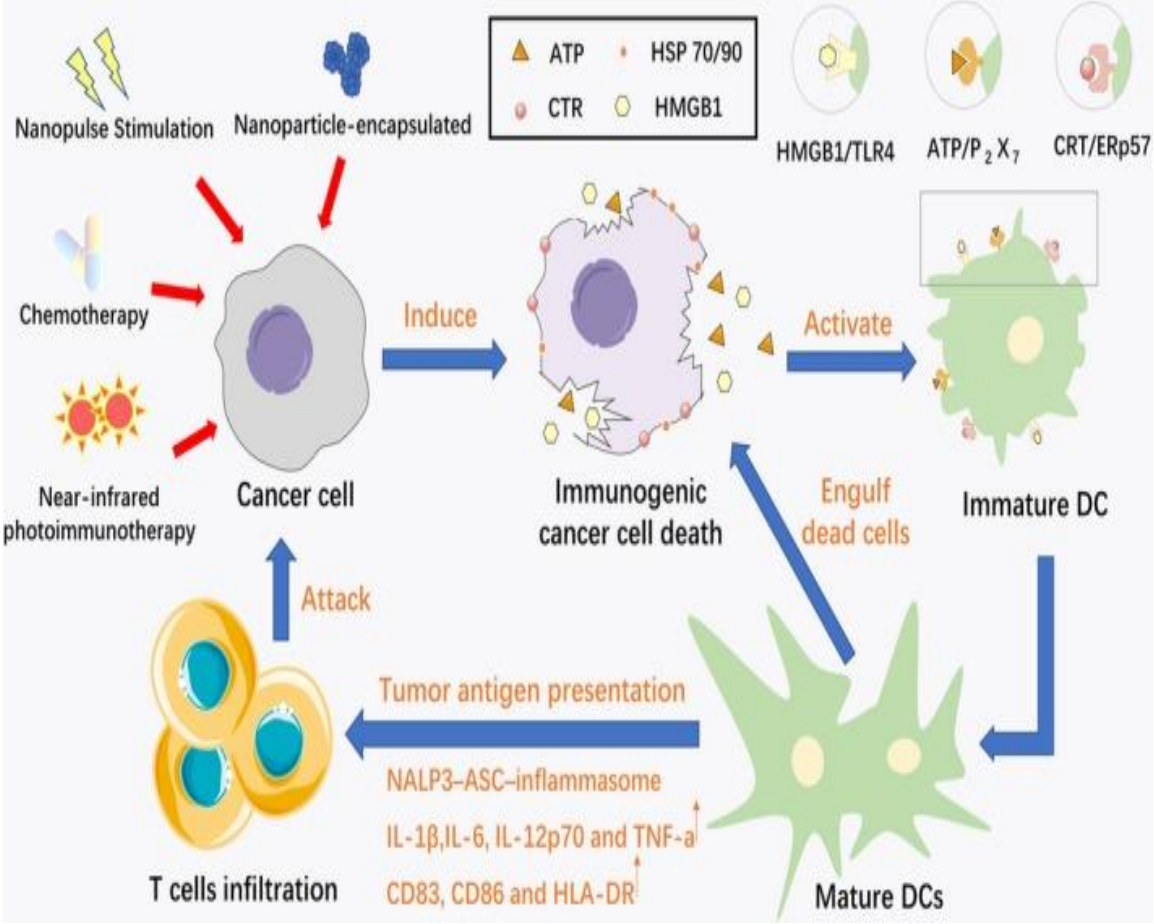

Figure 2. The mechanism of immunogenic cell death. After the induction of ICD, chronic exposure of damage-associated molecular patterns (DAMPs) on cancer cells attracts receptors and ligands on dendritic cells (DCs) and activates immature DCs to transition to a mature phenotype. CRT/ERp57 provides an 'eat me' signal that promotes phagocytosis of the cell by DCs;Extracellularly secreted adenosine triphosphate(ATP) is regarded as a 'find me' signal. The binding of high mobility group B1 to Toll-like receptor 4 (TLR4) and the expression of HSP70/90 have immunostimulatory properties that promote the processing of phagocytic cargo in DCs and

accelerate the engulfment of antigenic components by DCs, which consequently stimulate specific T cell responses and the killing of more cancer cells

Source: Zhou J, Wang G, Chen Y, Wang H, Hua Y, Cai Z. Immunogenic cell death in cancer therapy: Present and emerging inducers. J Cell Mol Med. 2019;23(8):4854-4865.

Moreover, HMGB1 can be excreated by stressed cells via an unknown pathway, not involving the endoplasmic reticulum[39]. HMGB1 is a mobile chromatin protein that acts as a DNA chaperone, by binding DNA transiently and bending it reversibly. HMGB1 is constitutively expressed in almost all cell types, and in order for it to act as a DAMP it must relocate extracelllarly. In response to exposure to stressful conditions cells not only release HMGB1 but also produce heat shock proteins (HSP). Thus, binding of HMGB1 to Toll-like receptor 4 (TLR4) and the expression of HSP70/90 have immunostimulatory properties that promote the processing of phagocytic cargo by DCs[44]. Consequently, via antigen presentation, DCs stimulate specific T cell responses that kill more cancer cells. The induction of ICD eventually results in long-lasting protective antitumor immunity. As already mentioned, DAMPS are not accessible by the immune system under physiological conditions but are released or exposed on the outer leaflet of the plasma membrane during cytoprotective stress responses or upon cell death. These observations have encouraged the increased usage of ICD-associated DAMPs as predictive/prognostic biomarkers[45]. Thus, it is clear that Immunogenic Cell Death (ICD) contrasts other forms of cell death that do not elicit any immune response or even mediate immune tolerance (Tolerogenic Cell Death; TCD). However, it needs to be clarified that ICD indicates a functionally peculiar type of apoptosis and regulated cell death such as necroptosis[41] (Figure 3).

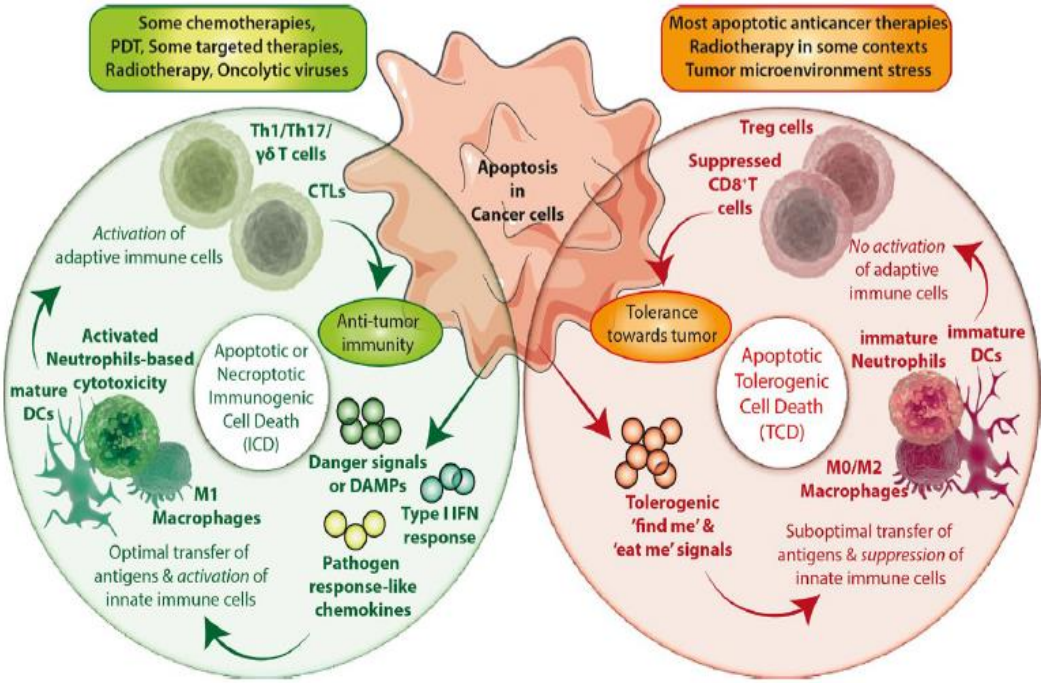

Figure 3. Schematic explanation of immunological patterns of apoptosis and their effects on tumour immunity or tolerance. DAMPs, damage-associated molecular patterns; DCs, dendritic cells; IFN, interferon; M0, immature macrophages; M1, type-1 polarized macrophages; M2, type-2 polarizedmacrophages; PDT, photodynamic therapy; Th1, type-polarized T cells; Th17, IL17A-producing T cells; Treg, regulatory T cells

Source: Garg AD, Agostinis P. Cell death and immunity in cancer: From danger signals to mimicry of pathogen defense responses. Immunol Rev. 2017;280(1):126-148.

4. Inducing ICD during anticancer treatment

The host immune system is continuously exposed to dying cells and has evolved to distinguish between cell death events signaling potential threats and physiological apoptosis that is tolerated. Tumors can use this distinction to their advantage, promoting apoptotic death of cancer cells to induce tolerance and evasion of immunosurveillance. On the other hand, stimuli that cause immunogenic death of cancer cells can induce an effective anti-tumor immune response. The presence of multiple inhibitory mechanisms in the same tumor microenvironment argues that combinatorial therapies may be advantageous, several of which are currently in clinical testing[46]. Several anticancer agents that have been successfully employed in the clinic for decades, including various chemotherapeutics and radiotherapy, can elicit ICD.

It is now clear that naive tumor cells can be effectively eliminated by the immune system except for those that successfully dodge the immune attack and establish an immunosuppressive microenvironment. As already mentioned, mobilizing and stimulating the immune system against cancer cells is one of the most effective ways to fight against cancer recurrence and metastasis. Creating an immunogenic microenvironment with the stimulation of the body's cytotoxic lymphocytes is of ultimate importance in the eradication of tumor cells. Cytokines like IFN-γ or TNF are key players in this fight[47]. Different types of chemotherapeutic agents promote cytokine response through ICD induction. ICD is further defined as a cell death that stimulates immune responses against neo-antigens exposed by dying or dead cells. The induction of ICD eventually results in long-lasting protective antitumor immunity[48]. Crucial DAMPs which can induce ICD include calreticulin (CRT), high mobility group box 1 (HMGB1), adenosine-5'-triphosphate (ATP) and heat shock proteins (HSP) 70 and 90, as already mentioned above. These DAMPs bind to innate immunity receptors, recruit and activate immune cells. Finally, they lead to ingestion by professional phagocytes and presentation of neo-antigens to T lymphocytes which further mount an anti-tumor reactive immune response.

The activity of DAMPs elicited during ICD is endoplasmic reticulum (ER) stress-dependent, either accompanied or triggered by reactive oxygen species (ROS)[49]. ICD inducers can be divided in two types. Type I ICD inducers are modalities that induce cell death via non-ER associated targets and danger signaling via ER stress; however this split in targeting might compromise their ability to fully target the ER. On the other hand, Type II ICD inducers selectively target the ER to induce both cell death as well as danger

signaling thereby causing ICD-associated immunogenicity in an ER-focused manner[50]. In other words treatments that indirectly initiate an ER stress response are considered to be type I ICD inducers, such as anthracyclines that target cytosolic or nuclear proteins, causing ER stress as a downstream effect. Treatments that are directly linked to ER stress are type II ICD inducers, such as photodynamic therapy (PDT) or oncolytic viruses that target the ER to trigger cell death[47]. The induction of ICD is regarded as stressor-dependent because ER stress and ROS production are the required components for the exposure of different DAMPs.

Recent evidence describes that activation of ER stress pathways also known as the unfolded protein response (UPR), and specially, the PERK-mediated arm of the UPR is vital for the vast majority, if not all, the scenarios where ICD occurs[51]. The endoplasmic reticulum (ER) acts as a moving organelle with many important cellular functions. As the ER lacks sufficient nutrients under pathological conditions leading to uncontrolled protein synthesis, aggregation of unfolded/misfolded proteins in the ER lumen causes the unfolded protein response (UPR) to be activated. The UPR is capable of recognizing the accumulation of unfolded proteins in the ER. The protein response enhances the ability of the ER to fold proteins and causes apoptosis when the function of the ER fails to return to normal. In different malignancies, ER stress can effectively induce the occurrence of autophagy in cells because malignant tumor cells need to re-use their organelles to maintain growth. Both ER stress and UPR activation are commonly reported in many different metabolic diseases and cancers. Information obtained from high throughput technologies has substantially improved our understanding of the UPR. During the accumulation of misfolded proteins in the ER lumen, the UPR response promotes the production of reactive oxygen species (ROS) in the endoplasmic reticulum. UPR is the basis of the pro-apoptotic mechanisms of certain anti-cancer patterns. Both autophagy and UPR signaling pathways are thought to be a strategy for cell self-protection. However, if the intensity or duration of cellular stress increases, these pathways will instead activate the mechanism of cell death[52]. Dying cells release or flag certain molecules on their outer plasma membrane that are functionally not immunogenic within cells, but if these molecules are released extracellularly or displayed at the cell surface, they can initiate an immunological response. Several sources indicated that ER stress and ROS synergistically activate danger signaling pathways that contribute to the mobilization of DAMPs to the extracellular space. Hence, ROS production and ER stress are crucial for ICD and, eventually, drive efficient antitumor immunity and this is the reason why components of the ER stress machinery may constitute clinically relevant druggable targets for the induction of ICD[53].

In this review, known agents inducing ICD, being beneficial for antitumor immunity, are presented. To date, several screening studies have been carried out to discover bona fide ICD inducers and reveal the inherent capacity of a wide variety of drugs to induce cell death-associated exposure of danger signals and bring about *in vivo* anti-cancer immune responses. Only a small, yet diverse, collection of anti-cancer therapies, whether chemotherapeutic drugs (e.g., anthracyclines, oxaliplatin, bortezomib) or physical modalities [e.g., radiotherapy, hypericin-based photodynamic therapy (Hyp-PDT)], have been shown to induce bona fide ICD. Summarizing the main chemotherapeutics that induce ICD, the recent experimental data, stirred up by the main bibliographic review, will be in parallel presented.

4.1. Type I ICD inducers

Conventional cytotoxic chemotherapeutics are generally classified according to their mechanism of action as follows: (1) alkylating agents that cause destabilization and breakage of the DNA strands during replication and eventually death of the cell (e.g., cyclophosphamide and oxaliplatin)[54], (2) antimetabolites that interfere with DNA replication and therefore cell division and tumor growth (e.g., 5-fluorouracil, gencitabine and mitoxantrone)[55], (3) topoisomerase inhibitors that interfere with the DNA unwinding process during DNA replication and transcription (e.g., irinotecan)[56], (4) cytotoxic antibiotics that kill cancer cells via excessive production of ROS and DNA intercalation (e.g., anthracyclines and bleomycin)[57], (5) microtubule poisons that inhibit tubulin polymerization/depolymerization and cause mitotic arrest (e.g., paclitaxel)[58]. The immunogenic effects of these agents were largely neglected in studies that mostly utilized cell cultures and immune-deficient animal models. In the past, promising anticancer agents were often moved forward into clinical trials without a thorough analysis of their immune modulatory effects. Most attention was paid to the common side effects of myelosuppression and lymphopenia of conventional chemotherapy, rather than chemotherapy-induced antitumor immune response[59].

Starting with alkylating agents, it is now considered that they can trigger immune responses by directly affecting immune cells. Cyclophosphamide, which belongs to the family of nitrogen mustards, is one of the most studied agents that reinstates the activity of T and NK cells by ablating regulatory T cells (Tregs) in the tumor microenvironment (TME). Low doses of cyclophosphamide in advanced cancer patients induced a profound and selective reduction of circulating regulatory T cells, associated with suppression of their inhibitory functions on conventional T cells and NK cells leading to a restoration of peripheral T cell proliferation and innate killing activities[60]. In 2011, cyclophosphamide was shown to induce widespread tumor apoptosis with strong immunogenic features[61]. Two types of actions relevant to the induction of antitumor immunity *in vivo* were observed. The effect on dendritic cell (DC) homeostasis, mediated by endogenous type I interferons (IFN-I), leading to the preferential expansion of CD8α+ DC[62], and the induction of tumor cell death with clear-cut immunogenic features capable of stimulating tumor infiltration, engulfment of tumor apoptotic material, and CD8 T-cell cross-priming by CD8α+ DC. Two observations were made: first, the translocation of CRT on the dying cell membrane as an “eat me” signal for DCs paralleled by the down-regulation of the “don't eat me” signal CD31 after treatment with the *in vitro* active cyclophosphamide analogue MAFO. Second, the release of soluble factors, among which HMGB1, which promoted the activation and survival of CD8α+ DC.

Platinum derivatives like oxaliplatin do not only have direct cytotoxic effect but also indirectly activate the immune system through induction of ICD. When tumor cells are exposed to oxaliplatin, HMGB1 is released, which activates DCs in a Toll-like receptor 4(TLR4)-dependent manner. While searching for the TLRs that might be involved in the immune response against dying tumor cells, TLR4 expression by DCs was found to be a prerequisite for efficient antigen presentation of tumor antigens furnished by dying cancer

cells. Both the release of HMGB1 by dying tumor cells and the TLR4–myeloid differentiation primary response protein-88 (MyD88) signaling pathway were required for the immune response against dying tumor cells and also for increasing the efficacy of anticancer chemotherapy and radiotherapy in mice[63]. Additional studies in colorectal cancer cells demonstrated that exposure to oxaliplatin caused expression of immunogenic signals prior to apoptosis. This activates the innate immune system and results in T-cell interferon production and interaction with TLR4 of dendritic cells creating an in situ tumor vaccine. Patients with mutant TLR4 genes demonstrated decreased response to oxaliplatin in metastatic cancer treatment with poorer disease free survival. Even the loss of a functional TLR4 allele was linked to decreased survival in colorectal cancer patients treated with oxaliplatin[64]. Oxaliplatin also induced an ER stress response that triggered the translocation of CRT from the ER to the plasma membrane via the PERK/elF2a/caspase 8/Bap31 axis. This involved a complex signal transduction pathway, including an ER stress response, the sub-apoptotic activation of caspase-8 and the exocytosis-dependent co-translocation of CRT, together with another ER protein, ERp57, to the outer surface of the plasma membrane[65]. With the release of CRT, also HMGB1 needs to be produced to achieve ICD. Cisplatin is another platinum agent used to treat a number of cancers including lung cancer, genitourinary cancer, breast or head and neck cancer. Both oxaliplatin and cisplatin were equally efficient in triggering HMGB1 release[65]. However, oxaliplatin, but not cisplatin, stimulated pre-apoptotic CRT exposure in a series of murine and human colon cancer cell lines. Immunogenicity of cisplatin and oxaliplatin are different, despite their similarities in the induction of immunogenic cell death. CRT induction may be a vital immunogenic mechanism causing reduced efficacy of cisplatin in colorectal cancer patients[64]. Moreover, there is no clear structure-function relationship to assist the prediction of ICD inducers. Thus, even though cisplatin and oxaliplatin exhibit considerable structural overlap and are capable of triggering ICD, the latter but not the former drives *bona fide* ICD[66]. Recently, the levels of ICD-associated DAMPs induced by chemotherapeutics commonly used in the clinical practice against non-small cell lung cancer (NSCLC) and the association of these DAMPs with apoptosis and autophagy were investigated and among cisplatin, carboplatin, etoposide, paclitaxel and gemcitabine, the first induced the highest levels of ICD-associated DAMPs[67]. Moreover, building on a Principal Component Analysis (PCA), a mathematical integration of ICD-associated DAMPs was created in an Index of Immunogenicity (IndImmunog) reflecting the immunogenic potential of each treatment. Cisplatin-treated cells showed the highest IndImmunog, while etoposide was the less immunogenic and the more pro-autophagic treatment[67].

As for antimetabolites, the main representatives that have been investigated for their ability to trigger ICD are gemcitabine, 5-fluorouracil or mitoxantrone. Gemcitabine, a nucleoside analogue widely used for treating breast cancer, non-small cell lung carcinoma (NSCLC), and pancreatic cancer, markedly reduced the number of myeloid suppressor cells (Gr-1+/CD11b+ cells) in the spleen of tumor-bearing mice without reducing the numbers of other immune cells[68]. Importantly, gemcitabine was capable of stimulating cross-priming of CD8+ T cells, increasing the number of immunostimulatory tumor-associated macrophages (M1 TAMs), and depleting circulating myeloid-derived suppressor cells

(MDSCs) in mouse models[59]. 5-Fluorouracil (5-FU) also depleted MDSCs in mouse models and increased the frequency of tumor infiltrating CTLs ,CD8+ T cells and natural killer (NK) cells. The study of randomized clinical trials showed how the presence of tumor infiltrating CTLs in colorectal cancer tissues administered 5FU related to clinical outcomes. In locally advanced rectal cancer the combination of chemotherapy and radiotherapy led to an increase in CTLs with a favorable therapeutic response[69]. Mitoxantrone, approved for use in acute myeloid leukemia and as second line treatment for prostate cancer, has been reported to trigger immunogenic cell death (ICD) in animal model studies. The drug induced typical characteristics of DAMP release, including increased exposure of calreticulin, and extracellular release of ATP and HMGB1 protein. Mitoxantrone also enhanced phagocytosis by dendritic cells. Further analysis revealed that mitoxandrone triggered ICD by activating eukaryotic initiation factor 2α (eIF2α) via PERK/GCN2 upregulation in prostate cancer cells[70]. In summary, it seems that the ICD-associated DAMPs for both gemcitabine and 5FU are HMGB1 and ATP secretion, while for mitoxantrone cell-surface CRT and ERp57 are additionally implicated.

Cytotoxic antibiotics, such as anthracyclines (doxorubicin epirubicin, idarubicin) or bleomycin, seem to have an immunostimulatory effect due to their cytotoxicity, however, through different pathways involving autophagy, ER stress response, and type I IFN response[59]. Doxorubicin belongs to the most important chemotherapeutics for the treatment of breast cancer. Thus, it has been studied for its immunostimulatory effects. Firstly, in anthracycline-induced ICD, calreticulin exposure obligatorily relies on the establishment of a pre-mortem ER stress response centered around the phosphorylation of eukaryotic translation initiation factor 2A(EIF2A)[71]. Secondly, ATP secretion requires the induction of autophagy[72]. In addition, type I IFN production stems from toll-like receptor 3 (TLR3) signaling[73]. Anthracyclines stimulate the rapid production of type I interferons (IFNs) by malignant cells after activation of the endosomal pattern recognition receptor Toll-like receptor 3 (TLR3). By binding to IFN-α and IFN-β receptors (IFNARs) on neoplastic cells, type I IFNs triggered autocrine and paracrine circuits that result in the release of chemokine (C-X-C motif) ligand 10 (CXCL10)[40]. Moreover, both doxorubicin and idarubicin treatments caused apoptosis in the cancer cell lines studied and the dose required to trigger ICD was generally higher than the dose needed to achieve cytotoxicity[48].

Proteasome inhibitors are drugs that block the action of proteasomes, cellular complexes that break down proteins. They have been studied in the treatment of cancer and three of them are approved for treating multiple myeloma[74],[75]. Multiple mechanisms are likely to be involved, but proteasome inhibition may prevent degradation of pro-apoptotic factors such as the p53 protein, permitting activation of programmed cell death in neoplastic cells upon suppression of pro-apoptotic pathways[75]. For example, bortezomib causes a rapid and dramatic change in the levels of intracellular peptides. During tumor development, cancer cells have to cope with harsh conditions that trigger ER stress. Thus, unfolded protein response (UPR) activation constitutes an important hallmark of several human cancers that endow cancer cells with the ability to acquire essential characteristics required for tumor progression[76].. Similar to bortezomib, carfilzomib another proteasome inhibitor used in the treatment of MM, has also shown to induce the exposure of CRT in different MM cell lines[77,78]. In the particular case of MM, their exacerbated secretory phenotype leaves these

cells heavily reliant on the survival arm of the UPR. Therefore, as plasma cell development and survival strongly relies on an intact UPR[79], it does not seem unusual that UPR activity increases with MM progression. This feature explains why proteasome inhibitors, have shown a prominent clinical efficacy in the treatment of MM [77,80]. It is important to point out that MM is a genuine example where the immune system is compromised. However, treatment with bortezomib induced apoptosis with similar kinetics and promoted dendritic cells (DC) maturation. The surface expression of molecules involved in immune activation, namely calreticulin (CRT), heat shock proteins (HSP) 90 and 70 increased in dying cells[81].

Moreover, there is some evidence that microtubule-targeting agents including taxanes and vinca alkaloids (which are commonly used for the treatment of multiple carcinomas) can stimulate ICD. Treatment of ovarian cancer cells with Paclitaxel was followed by the emission of DAMPs, such as calreticulin (CRT) exposure, ATP secretion, and HMGB1 release *in vitro* and elicited significant antitumor responses in tumor vaccination assays *in vivo*[82]. Paclitaxel-induced TLR4 signaling was essential to the release of DAMPs, which led to the activation of NF-κB, thus exposing CRT on the cell surface. Paclitaxel induced endoplasmic reticulum stress, which triggered protein kinase R–like ER kinase activation and eukaryotic translation initiation factor 2α phosphorylation independent of TLR4. Paclitaxel chemotherapy induced T-cell infiltration in ovarian tumors of the responsive patients[83], CRT expression in ovarian tumors also correlated with patients' survival and patient response to chemotherapy[84]. These findings suggest that the effectiveness of paclitaxel relied upon the activation of antitumor immunity through ICD via TLR4 and highlighted the importance of CRT expression in cancer cells as an indicator of response to paclitaxel chemotherapy in ovarian cancer[82].

Except from classic ICD inducers cardiac glycosides (CGs), like digoxin, digitoxin and shikonin have been studied for the successful induction of ICD. Cardiac glycosides are primarily used to restore cardiac rhythm by targeting calcium regulation to force contractions. *In vivo* experiments with digoxin and digitoxin from identified cardiac glycosides (CGs) as exceptionally efficient inducers of immunogenic cell death, an effect that was associated with the inhibition of the plasma membrane Na+- and K+-dependent adenosine triphosphatase (Na+/K+-ATPase)[85],which is their main mechanism of action. Moreover, cancer cells succumbing to a combination of chemotherapy plus CGs could vaccinate syngeneic mice against a subsequent challenge with living cells of the same type. Finally, retrospective clinical analyses revealed that the administration of the CG digoxin during chemotherapy had a positive impact on overall survival in cohorts of breast, colorectal, head and neck, and hepatocellular carcinoma patients, especially when they were treated with agents other than anthracyclines and oxaliplatin[86,87]. CGs increased the antineoplastic effects of DNA-damaging agents in immunocompetent but not immunodeficient mice[86].

Another approach to killing cancer cells is the application of radiotherapy. The cytotoxic effect of radiation on tumor cells lies on the production of DNA double-strand breaks followed by some form of cell death, including apoptosis, necrosis autophagy, mitotic catastrophe, or replicative senescence[88]. Radiotherapy can induce a type of cell death in a subset of susceptible tumor cells, which can then activate antigen uptake, cell maturation and presentation by antigen-presenting cells (APCs). This is identified by three main

hallmarks on tumor cells; calreticulin exposure, ATP release and HMGB1 release, which are essential for the promotion of CD8+ T-cell anticancer responses. Primed CD8+ T cells contribute to subsequent residual tumor cell elimination in the tumor bed as well as non-irradiated lesions distant from the radiated field [89]. This is called the abscopal effect (ab-scopus, away from the target). Molecules that can alter the lytic capacity or enhance the sustainability of effector CTLs or NK cells are likely candidates for promoting this type of effect[90]. Its occurrence provides another proof of principle for the involvement of the immune system as a result of radiotherapy. In other words, local radiation triggers systemic effects that can be used in combination with immunotherapy to induce responses outside the radiation field[88]. Most studies combine the standard radiation dose and regimens indicated for the given disease state, with novel cancer immunotherapies.

4.2. Type II ICD inducers

By the 1980s, researchers started to report more specific observations regarding the therapeutic impact of cancer cell immunogenicity. For example, the ability of curative hyperthermia to cause the (heat-shock based) generation of circumstantial anti-tumor immunity or the increase of the immunogenicity of cancer cells by the application of hydrostatic pressure[91,92]. Gradually these observations led to the study of new methods applied to cancer tissues and revealed induction of immunogenic cell death in a common pathway by the Type II inducers, agents that induce ICD through a "focused" ER stress effect. To date, hypericin-based photodynamic therapy (Hyp-PDT)[3593], oncolytic adenovirus[94], oncolytic coksackie virus B3 (CVB3)[95], oncolytic Newcastle disease virus(NDV)[96,97] have been studied.

Mechanistically, it has been shown that hypericin is a photosensitizer that promotes substantially enhanced ROS generation upon excitation by specific wavelength thus, resulting in a targeted ROS-based ER stress. When the photosensitive dye accumulates in the cancerous tissue, to initiate its elimination, multiple signaling pathways are activated in cancer cells, which could give rise to all several cell death modalities, at least *in vitro*. Moreover, when cancer cells are treated with hypericin-based PDT (Hyp-PDT), they surface-expose both HSP70 and calreticulin (CRT). Induction of CRT exposure was not accompanied by co-exposure of ERp57, but this did not compromise the ability of the exposed CRT to regulate phagocytosis of Hyp-PDT-treated cancer cells by dendritic cells[93]. Simultaneously, PDT is capable of eliciting various effects in the tumor microenvironment thereby affecting tumor-associated immune cells and by extension, leading to migration of various immune cells (e.g. neutrophils) into the treated site. In both pre-clinical as well as clinical settings, PDT appears capable of activating "anti-tumor adaptive immunity". Therefore, it seems that PDT is unique among other approved therapeutic procedures in generating a microenvironment suitable for the development of systemic anti-tumor immunity[35].

Oncolytic viruses are self-replicating, tumor-selective viruses, with an ability to preferentially infect and kill cancer cells. As the infected cancer cells are destroyed by oncolysis, they release new infectious viral particles or virions to help destroy the remaining tumor. Oncolytic viruses are thought not only to cause direct destruction of the

tumor cells but also to stimulate host anti-tumor immune system responses. Robust viral replication in tumors provides immunologic damage-associated molecular pattern (DAMP) signals, augmenting the immunogenicity of the tumor microenvironment. Previous studies showed that several oncolytic viruses could induce adaptive antitumor immunity by tumor-specific CTL responses. The capacity of an oncolytic adenovirus (Delta-24-RGD) to trigger an antitumor immune response was tested in a syngeneic mouse model. The experiment included intracranial injection of mouse glioma cells into the brain of mice, followed by treatment of the tumors with intratumoral injections of the oncolytic adenovirus. The results indicated that adenovirus-infected cancer cells expressed not only PAMPs but also DAMPs [112]. Further research with adenoviruses showed that coding for CD40L mediates multiple antitumor effects including oncolysis, apoptosis, induction of T-cell responses, and upregulation of T(H)1-type cytokines[94]. In summary, the intra-tumoral necrosis (local effect) induced by oncolysis serves as a mechanism for activation of the innate and adaptive immune responses that will eventually result in the elimination of distant invasive cells, including metastases (systemic effect).

It is clear that pre-apoptotic exposure to CRT and ATP release, as well as post-apoptotic HMGB1, are required for immunogenic cell death induced by both type I inducers and type II inducers. The coxsakie virus CVB3 infection promoted similar effects on NSCLC cells. CVB3 treatment induced abundant surface exposure of CRT on A549 cancer cell lines, and active secretion of extracellular ATP was dose- and time-dependent. In addition, CVB3 infection resulted in substantial release of HMGB1[95].

The effect of Newcastle disease virus (NDV) was investigated in the orthotopic glioma, syngeneic murine GL261 model. Seven days after tumor induction, mice received NDV intra-tumorally. They demonstrated immunogenic cell death (ICD) induction in GL261 cells after NDV infection, comprising calreticulin surface exposure and release of HMGB1. They also observed absence of secreted ATP[96]. Oncolytic NDV, tested in melanoma cells, also induced CRT exposure, release of HMGB1 and HSP70/90 as well as secretion of ATP[98].

Among the group of oncolytic viruses, Measles virus (MV) and Herpes simplex virus (HSV) are also able to induce ICD. Upon testing MV against human melanoma cell lines, it was clear that MV triggered cell death causing inflammatory response through release of type-1 interferons and HMGB1 as a danger signal. Thus, MV-mediated melanoma cell death was capable of stimulating a melanoma-reactive adaptive immune response[99]. Another study indicated that HSV-1 was able to lead squamous cell carcinoma cells to apoptosis, along with the release of both HMGB1 and ATP and the exposure of CRT[100].

5. Clinical applications and challenges

It is widely accepted that the administration of chemotherapy and radiotherapy or their combination to treat solid tumors induce different clinical responses with an impact on survival benefit of cancer patients[101]. Most chemotherapeutic drugs or radiotherapy mediate their cytotoxic effects by the induction of apoptosis, which is generally considered to be non-inflammatory and non-immunogenic. However, it has been proposed in clinical

trials that danger signals released by dying cells following chemo/radiotherapy could induce HMGB1/TLR4-dependent, antigen-specific T-cell immunity. Furthermore, DAMPs such as, CRL exposure, ATP secretion and type I interferon production, seem to be required for immunogenic cell death induced by chemotherapeutics or radiotherapy[102]. There is accumulating evidence to support the novel concept that radiotherapy and/or chemotherapy can induce immunogenic cell death and trigger uptake of antigenic components by DCs, which stimulate antigen-specific CTLs and production of tumor-specific monoclonal antibodies in murine models.

Recently, after investigating mechanisms underlying immunogenic cell death induced by chemo/radiotherapy, we could potentially define two clinical applications. One would be prognostic, that is, to foretell the extent of benefit of chemo/radiotherapy by measuring surrogate markers such as HMGB1 or calreticulin (CRL). The other application would be therapeutic, that is for treatment, which improves the impact of the chemo/radiotherapy by sequentially combining agents and other immune-activating therapies in patients with the biomarker profile of potential good responders.

The clinical existence of ICD has been proven through retrospective analysis involving cancer patient's survival/therapy-responsiveness data. For example, chemo/radiation was shown to induce upregulation of local HMGB1 with significant variations among Esophageal Squamous Cell Cancer patients; patients with high HMGB1 expression had better OS than patients with weak HMGB1 expression[103]. Moreover, Circulating Myeloid-derived suppressor cells (MDSCs) levels are increased in the blood of patients with several types of cancer and can induce profound suppression of T-cell and NK-cell functions. As demonstrated from several trials, chemotherapeutic agents such as gemcitabine and 5-fluorouracil can downregulate MDSC frequencies and may, therefore, add to their clinical efficacy [103]. Clinical evidence suggests a correlation between disease outcomes and CRT cell-surface translocation and CD47 surface expression in cancer cells. For example, deficient CRT exposure in colorectal tumor cells is associated with loss of TILs and poor prognosis[104]. Doxorubicin markedly changed the immune infiltration of breast tumors and high infiltration of CTLs in breast tumors at diagnosis predicts favorable therapeutic response[105].

The relevance of immunogenic cell death in clinical settings lies in the ability of this process to reactivate pre-existing anticancer immunity. Some successful chemotherapeutics, notably anthracyclines and oxaliplatin, induce a type of cell stress and death that is immunogenic, hence converting the patient's dying cancer cells into a vaccine that stimulates antitumor immune responses. In addition, emerging applications of local radiotherapy as an immunologic adjuvant have provided radiation oncologists with a method for converting malignant cells into endogenous anticancer vaccines[89]. We have noticed from the study of the literature that in preclinical trials the only way to identify *bona fide* ICD inducers is through vaccination challenges. Tumor cells treated with ICD inducers prior to inoculation into immunocompetent mice protect mice from subsequent challenge with the same tumor[38]. Certain ICD inducers have been used in clinical treatments for various cancers.

Conventional cytotoxic drugs are often immunosuppressive and associated with drug resistance and tumor regrowth after a short period of tumor shrinkage or growth stasis. However, certain cytotoxic cancer chemotherapeutics, including cyclophosphamide,

doxorubicin, epirubicin, idarubicin, mitoxantrone, and oxaliplatin, can kill tumor cells by an immunogenic cell death pathway, which activates robust innate and adaptive anti-tumor immune responses and has the potential to greatly increase the efficacy of chemotherapy. The impact of ICD can be seen when immune competent mice are injected with tumor cells treated *ex vivo* with mitoxantrone, doxorubicin or idarubicin, which confers immunity against live tumor cell challenge on the opposite flank. Thus, the ICD drug-treated tumor cells immunize the host to the tumor and thus serve as an anti-cancer vaccine[106]. Other DNA-damaging agents, such as etoposide and mitomycin C, are non-immunogenic, and show little such vaccine activity when tested in the same experimental setting[67]. However, the immunogenicity of etoposide and mitomycin C becomes apparent when calreticulin is overexpressed or when protein phosphatase-1/GADD34 complex, a negative regulator of calreticulin exposure, is inhibited.

A key goal of ICD-based chemotherapy is to take advantage of the synergistic effects of combining tumor cell cytotoxicity with ICD-induced activation of the patient's immune system in order to eliminate tumor cells, in particular, tumor cells that may be resistant to conventional chemotherapy. Ideally, this would be achieved in a way that activates both the innate and the adaptive immune system and leads to tumor ablation with long-term anti-tumor immune memory. In this Review, we focus on the combination of ICD inducers and immunotherapy. One of the hallmarks of cancer progression is the induction of immunosuppression, which allows the tumor to evade detection and/or elimination by the immune system. Tumors suppress the adaptive immune response at the level of antigen presentation by downregulating the expression of tumor antigens, the antigen processing machinery, and MHC class I and II molecules. In addition, tumor cells can drive the expansion of immunosuppressive myeloid derived suppressor cells (MDSCs). MDSCs can directly suppress T cell responses, and indirectly promote immune suppression through the induction of regulatory T cells (Tregs). Given that the benefits of ICD require antigen presentation by DCs and a functional T cell response, patients that have suppressed immune systems may exhibit reduced responses to chemotherapeutics that induce ICD. These patients may benefit from combinatorial therapies that combine inducers of ICD with immune-stimulation or targeting of immunosuppressive populations.

Thus, the combination of immunostimulating anticancer therapy and immune checkpoint blockade has become crucial[6]. Antagonist antibodies used to block inhibitory receptors allow for activation of anti-tumor immune responses that would otherwise be strongly suppressed. Cytotoxic drugs may be particularly effective when administered in the context of checkpoint blockade, when the tumor cells are already under T cell attack and may be more susceptible to drug toxicity. Cytotoxic drugs administered at this point may also kill tumor cells that escape T cell attack, and may block the increases in Tregs and other immune suppressive cells that often follow immune stimulation[107]. Checkpoint blockade applied following a cycle of chemotherapy is also expected to be effective, in particular for drugs that induce ICD: as chemotherapy-induced tumor cell cytotoxicity and anti-tumor immune responses wane and pro-tumor immune responses rebound, the checkpoint inhibitors may suppress pro-tumor immune responses and thereby prolong, and perhaps augment immune responses activated by the immunogenic chemotherapy[108].

Strategies with three distinct checkpoint blockers, namely the CTLA4-targeting mAb ipilimumab and the PD-1 targeting mAbs nivolumab and pembrolizumab are currently

used worldwide as standalone immunotherapeutic interventions in different malignancies. As expected, due to their role in reversal of inhibition of tumor immunity, administration of anti-CTLA-4 or anti-PD-1/PD-L1 monoclonal antibodies (mAbs) leads to activation of the immune system[109]. Although, most conventional anticancer regimens mediate killing of tumor cells mainly by activating apoptosis, an immunosuppressive or even tolerogenic cell death process, as previously mentioned, a selected class of cytotoxic agents (e.g., anthracyclines) can cause an immunogenic form of apoptosis in tumor cells and consequently, these dying cells can induce an effective antitumor immune response[43]. Recent studies showed that immunogenic cell death (ICD) improves T cell responses against different tumors, indicating that ICD may further augment antitumor immunity elicited by anti-PD1 agents. Theoretically, we can assume that ICD converts dying cancer cells into a therapeutic vaccine and stimulates antitumor immune responses.

Yet, more than half of treated patients do not respond to immune checkpoint blockade therapy, even if combinations of blocking antibodies were used. Malignant cells develop different strategies to evade the immune system and create an environment that supports their proliferation. To escape immune surveillance, they take advantage of negative feedback mechanisms initially evolved to prevent immunopathology. These include inhibitory cytokines such as IL-10 and tumor growth factor (TGF)-β, inhibitory cell types such as regulatory T cells (Tregs), regulatory B cells (Bregs), and myeloid-derived suppressor cells (MDSCs), metabolic modulators such as indoleamine 2,3-dioxygenase (IDO), and inhibitory receptors such as PD-L1/2 as already mentioned [110]. Moreover, cancer cells bearing inherent genetic instability form new antigens (neoantigens), which have not been previously recognized by the immune system. To avoid immune surveillance targeting immunogenic cancer antigens including neoantigens, cancers acquire resistance by selecting less-immunogenic variants and establishing an immunosuppressive environment using immunosuppressive elements to become clinically "overlooked cancers"[32].

6. Conclusions and Perspectives

For many decades the scientific community has been struggling to fight cancer. This is a very difficult task as the biggest burden lies in the discovery of selective treatments targeting tumor cells mainly, in order to alleviate the side effects connected to killing of neighboring normal cells. In clinical practice, the effectiveness of a treatment varies considerably depending on tumor's type, genetic background, heterogeneity and microenvironment. Thus, solid tumors often become resistant and non-responsive to conventional therapies.

A significant parameter contributing to high mortality and recurrence rates among cancer patients is the ability of tumor cells to escape from the immune system. It is obvious that tumor cells have developed a number of strategies to escape immune surveillance including their ability to avoid apoptosis and cell death. This is considered one of cancer's hallmarks and it is well established that cancer cells have multiple mechanisms to subvert cell death pathways and can thereby become resistant to immune attack.

During the last decade, as several signaling pathways have been defined for specific forms of cell death, our conception of Programmed Cell Death (PCD) has expanded beyond apoptosis to encompass additional modes, including necroptosis and pyroptosis which are notable for their diverse roles in engaging the immune system. Concurrently, treatments that activate the immune system to combat cancer have been successfully applied in the clinic. Therefore, new perspectives on the role of programmed cell death in cancer therapy are being drawn up due to emerging evidence that induction of alternate death pathways could improve therapeutic outcomes.

Apoptosis has long been considered a non-immunogenic or even tolerogenic process, implicated in immune suppression and promotion of tumor growth. On the other hand, necrosis and necroptosis have been shown to play a key role in inflammation and immune related processes, respectively. In particular, necroptosis is associated with inflammatory cytokine production and priming of adaptive immune responses. Moreover, the new concept of "Immunogenic Cell Death" (ICD) has challenged the traditional view and has granted apoptosis, also, with immunogenic abilities. Certain chemotherapeutic agents induce ICD *in vitro* and *in vivo* through PCD (apoptosis or necroptosis), thereby enhancing the immunogenicity of the tumor. Great efforts are also being devoted to the development of combinatorial regimens relying on the co-administration of conventional or targeted anticancer agents in conjunction with one or more protocols of immunotherapy. The clinical profile of anticancer chemotherapy based on ICD inducers may be considerably ameliorated by the concomitant administration of various immunostimulatory interventions, in particular checkpoint blockers.

Nevertheless, despite intense efforts and the spectacular progress made as to the elucidation of the ICD mechanisms and the effectiveness of the combined therapies, open questions still remain towards full exploitation of the immune response to a certain chemotherapy in the clinical practice. Unfortunately, the gold-standard approach to detect ICD relies on vaccination experiments involving immunocompetent murine models and syngeneic cancer cells, an approach that is incompatible with large screening campaigns. The mechanisms through which necroptosis engages inflammation and adaptive immunity should be fully elucidated, as well as its differences from apoptosis and necrosis. Therapies that preferentially trigger and maximize ICD through PCD in tumor cells should be specified in detail. For that, deep knowledge of PCD signaling is needed. It is certain that in evaluating the feasibility of incorporating ICD into existing immunotherapy regimens, focusing on necroptosis-inducing drugs in particular, more *in vivo* studies are required to assess tumor cell specificity and overall efficacy of these therapies.

In conclusion, considerable effort has been made to develop strategies to target cell death for clinical purposes while advances in tumor immunology have undisclosed some key mechanisms that represent the basis of therapeutic synergy or antagonism with other treatments. Certain chemotherapeutic agents can induce immunogenic cell death which in combination with immunotherapy determines cumulative immune stimulation to fight tumors. The presence of both prognostic and predictive biomarkers related to immunogenic death is necessary for this purpose. Though additional studies are still required to devise the most efficient strategies, the ensemble of results discussed herein definitely pave the way towards mechanism-based, rather than empirical, rationales for combination of specific chemotherapeutic agents with selective immunotherapeutic interventions, opening novel horizons for a far more effective management of cancer patients.

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