Trial watch: Immunogenic cell death induction by anticancer chemotherapeutics

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ABSTRACT

The expression “immunogenic cell death” (ICD) refers to a functionally unique form of cell death that facilitates (instead of suppressing) a T cell-dependent immune response specific for dead cell-derived antigens. ICD critically relies on the activation of adaptive responses in dying cells, culminating with the exposure or secretion of immunostimulatory molecules commonly referred to as “damage-associated molecular pattern” molecules. Only a few agents can elicit bona fide ICD, including some clinically established chemotherapeutics such as doxorubicin, epirubicin, idarubicin, mitoxantrone, bleomycin, bortezomib, cyclophosphamide and oxaliplatin. In this Trial Watch, we discuss recent progress on the development of ICD-inducing chemotherapeutic regimens, focusing on studies that evaluate clinical efficacy in conjunction with immunological biomarkers.

Introduction

The possibility that cancer cells might undergo an immunogenic form of regulated cell death (RCD) in response to selected stimuli has been proposed for the first time more than 10 years ago.1 Since then, considerable efforts have been dedicated to the elucidation of the molecular and cellular mechanisms underlying immunogenic cell death (ICD), defined as a functionally distinct form of regulated cell death that facilitates (instead of suppressing) an adaptive immune response specific for dead cell-derived antigens.1-7 Based on the available data, it is clear that ICD can facilitate T cell responses against a wide-spectrum of differentiation, over-expressed, and mutated tumor-associated antigens (TAAs).1-10,11 However, the predominance of a fraction of TAA-specific T cells in driving ICD-based immunity might be regulated by: (1) the spatiotemporal expression patterns of specific TAAs within a tumor,14-20 (2) the overall coverage of various TAAs by central or peripheral tolerance,21-23 (3) the overall avidity of the T cell receptor (TCR) for specific TAAs,24-32 and (4) the general cellular and metabolic health of effector or memory T cell fractions.33-38 Operationally, two experimental procedures have been established over the years to identify bona fide ICD inducers in vivo. First, bona fide ICD inducers display elevated efficacy against malignant cells growing in immunocompetent hosts, but are largely ineffective when the same tumors are established in immunocompromised animals.39-43 Second, cancer cells succumbing to bona fide ICD in vitro are able to vaccinate syngeneic immunocompetent hosts against a subsequent challenge with living cancer cells of the same type.1,3,39-44 Although the former approach (therapeutic setting) is rather convenient, it is intrinsically unable to discriminate between bona fide ICD inducers and molecules that exert other on-target immunostimulatory effects or drive off-target immunostimulation.45-47 Thus, the only gold-standard approach to identify immunogenic instances of cell death relies on the latter approach (vaccination setting).39 Since vaccination tests can only be performed with murine cancer cells and immunocompetent syngeneic hosts, however, surrogate approaches have been developed.3-39,46 On the one side, surrogate biomarkers of ICD can be measured in (human and murine) cancer cells responding to putative ICD inducers.2,44-48 On the other side, malignant cells succumbing to a putative ICD inducer can be fed to dendritic cells (DCs),2,44,52-55 followed by (1) phagocytosis assays,56-62, (2) assessment of activation markers on the DC surface (e.g., CD80, CD86, MHC Class II) and functional markers in conditioned media (e.g., interleukin-6 or IL6, IL1β, IL12p70),34,63-72, or (3) functional cross-
A number of mechanisms regulate the capacity of a particular agent to drive bona fide ICD and the ability of the host to perceive such an instance of cell death as immunogenic, and hence respond with potentially curative TAA-specific adaptive immunity. \(^3\) At the level of cancer cells, ICD depends upon the timely emission of a constellation of immunomodulatory damage-associated molecular patterns (DAMPs). \(^{40,83}\) In the case of chemotherapy-induced ICD, these include (but may not be limited to): (1) surface-exposed endoplasmic reticulum (ER) chaperones including calreticulin (CALR)\(^{84-86}\), (2) extracellular ATP; \(^{87-93}\) (3) extracellular high mobility group box 1 (HMGB1)\(^{13,92}\); (4) extracellular annexin A1 (ANXA1)\(^5\); (5) calreticulin (CALR)\(^84\), (6) extracellular nucleic acids. \(^7\) That said, ICD triggered by stimuli other than chemotherapy (e.g., radiation therapy, photodynamic therapy) is not necessarily associated with the same DAMPs. \(^{3,40,98,99}\) Moreover, new DAMPs underlying the immunogenicity of specific instances of RCD are continuously being uncovered (see below).

When emitted in a proper spatiotemporal pattern, in combination with TAAs, and in the context of a tumor milieu amenable to immune intervention, \(^83,100-104\) these DAMPs can efficiently recruit antigen-presenting cells including DCs to the tumor bed, facilitate the engulfment of dying cells or their corpses (along with their TAA-laden cargo) in the context of immunostimulatory signals (which promote DC maturation). \(^{13,41,44,93,99}\) Mature DCs acquire therefore the ability to cross-present processed TAAs to CD4\(^+\) and CD8\(^+\) T cells along with suitable co-stimulation, thereby resulting in engagement of TAA-specific immunity. \(^105-110\) Accordingly, RCD can no longer be perceived as immunogenic when: (1) the intracellular stress responses regulating the emission of ICD-associated DAMPs are pharmacologically or genetically ablated in cancer cells; or (2) when the molecular machinery dedicated to DAMP detection is inhibited or ablated. \(^13,44,84,91,93,97\) Moreover, ICD-driven immunity can no longer operate in the presence of general immunological defects, \(^111\) such as (1) an intrinsically low antigenicity of cancer cells, owing to low levels of TAAs or downregulation of MHC Class I molecules \(^112-119\); (2) an increased immunological tolerance of the host, secondary to increased amounts of immunosuppressive cytokines. \(^120-128\) or inhibitors of chemotaxis, \(^129-134\) increased tumor infiltration by immunosuppressive cell populations, \(^135-145\) or robust immune checkpoint activation; \(^126,130,136-138,146\) (3) a reduced persistence of TAA-specific CD8\(^+\) memory T cells due to peripheral tolerance; \(^21,23,144,147-149\) and (4) an intrinsically elevated resistance of cancer cells to lysis by immune effectors. \(^136,147,150,151\) Additional details about ICD-associated signaling pathways and resistance mechanisms can be found in various publications from us and others. \(^7-4,7,40,152-155\)

Of note, only a limited number of cell death inducers can elicit bona fide ICD, and this capacity cannot be predicted on the basis of structural or functional similarities. Thus, while cisplatin and oxaliplatin both induce RCD at least in part by forming inter- and intra-strand DNA adducts, \(^156\) only the latter induces ICD. \(^157\) Similarly, even though both melphalan and cyclophosphamide efficiently kill cancer cells by operating as DNA alkylating agents, only the latter drives ICD. \(^158\) In both examples, the ability of a specific agent (e.g., oxaliplatin, cyclophosphamide) but not one of its alike (e.g., cisplatin, melphalan) to drive ICD can be explained by the differential activation of ER stress (and hence differential exposure of CALR in the course of RCD). \(^100,157-159\) Well-established ICD inducers include commonly employed anticancer chemotherapeutics such as: (1) doxorubicin, an anthracycline approved by the US Food and Drug Administration (FDA) for treating acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), small cell lung carcinoma, breast carcinoma, neuroblastoma, lymphoma, thyroid carcinoma, soft tissue and bone sarcomas, multiple myeloma (MM), gastric cancer, ovarian carcinoma, transitional cell bladder carcinoma and Wilms tumor; \(^1,84,160-166\) (2) epirubicin, an anthracycline licensed for use in breast carcinoma patients; \(^1,84,167,168\) (3) idarubicin, an anthracycline generally employed against AML, \(^84,167-169\) (4) mitoxantrone, an anthrancenedione approved for the treatment of prostate carcinoma, AML, non-Hodgkin’s lymphoma (NHL) and breast carcinoma; \(^1,84,167,168\) (5) bleomycin, a glycopeptide antibiotic approved for the treatment of patients with NHL, testicular cancer, Hodgkin’s lymphoma, penile cancer and squamous carcinomas of the cervix, head and neck or vulva; \(^157,167,168\) (6) bortezomib, a proteasomal inhibitor approved for the therapy MM and mantle cell lymphoma (MCL); \(^171-181\) (7) cyclophosphamide, a DNA-alkylating agent approved for use in patients with chronic myeloid leukemia (CML), AML, ALL, chronic lymphocytic leukemia, MM, ovarian carcinoma, breast carcinoma, mycosis fungoides, lymphoma, neuroblastoma, and retinoblastoma; \(^177,182-191\) and (8) oxaliplatin, a platinum-derivative licensed for the therapy of advanced colorectal carcinoma in combination with 5-fluorouracil and folinic acid. \(^198,199\) 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. \(^191-199\)

Along the lines of our Trial Watch series, here we discuss recent preclinical and clinical advances in the development of ICD-inducing chemotherapeutic regimens. \(^200\) Several other interventions that trigger bona fide ICD, such as radiation therapy administered according to specific regimens, \(^94,201-203\) high hydrostatic pressure, \(^3,4\) oncolytic virotherapy \(^204-208\) and photodynamic therapy, \(^44,86,98,99\) are not discussed here in further detail.

**Recent preclinical developments**

A high amount of preclinical and/or translational studies on chemotherapy-induced ICD has been published since the latest Trial Watch dealing with this topic (April 2015). \(^50\) Of such an abundant scientific production from us and others, we found of particular significance the following works.

We documented that anthracycline-induced ICD critically relies on the release of ANXA1 by cancer cells, driving the late-stage phases of DC chemotaxis in a formyl peptide receptor 1 (FPR1)-dependent manner. \(^55\) Accordingly, a loss-of-function single-nucleotide polymorphism in FPR1 was found to be associated with poor prognosis in cohorts of breast and colorectal
carcinoma patients undergoing anthracycline or oxaliplatin-based chemotherapy.\textsuperscript{55} Moreover, we found that combining chemotherapy-induced ICD with short-term fasting or caloric restriction mimetics (CRMs)\textsuperscript{90} like hydroxyurea or spermidine, boosts the efficacy of mitoxantrone and oxaliplatin in mice, a therapeutic improvement that is accompanied by decreased tumor infiltration by immunosuppressive CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+} regulatory T (T\textsubscript{REG}) cells.\textsuperscript{209} Along similar lines, Di Biase et al. (from University of Southern California, Los Angeles, CA, USA) observed that the combination of doxorubicin with a fasting-mimicking diet strongly delays breast cancer and melanoma progression as it increases the amounts of tumor-infiltrating cytotoxic T lymphocytes (CTLs) while concomitantly decreasing the expression levels of the immunosuppressive enzyme heme oxygenase-1 (HMOX1; also known as HO1).\textsuperscript{210} We also discovered that cancer cells undergoing mitoxantrone-induced ICD trigger a pathogen response-like chemokine (PARC) signature characterized by the co-release of C-X-C motif chemokine ligand 1 (CXCL1), C-C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine ligand 10 (CXCL10) (or homologues thereof), in thus far mimicking bacterial or virus infected cells.\textsuperscript{97} Such a chemokine mixture is particularly efficient at recruiting neutrophils towards the dying cells (a process that appears to be evolutionarily conserved), paving the way to the CALR-dependent phagocytosis of dying cancer cells or corpses thereof, and the cytotoxic targeting of residual malignant cells.\textsuperscript{97} We characterized a naturally-occurring preclinical model of cancer that exhibits intrinsic resistance against mitoxantrone-induced ICD in vivo secondary to a defect in CALR expression,\textsuperscript{199} and we documented that anthracyclines and oxaliplatin can trigger a necrototic variant of ICD\textsuperscript{211-215} in cancer cells expressing receptor interacting serine/threonine kinase 3 (RIP3K) and the pseudokinase mixed lineage kinase domain-like (MLKL).\textsuperscript{215} Finally, we found that an engineered oncolytic vaccinia virus\textsuperscript{216} can induce ICD-dependent antitumor immunity, which can be further potentiated by the co-administration of ICD-inducing chemotherapy or immune checkpoint blockers (ICBs),\textsuperscript{216} and that pharmacological inhibition of signal transducer and activator of transcription 3 (STAT3)\textsuperscript{217} signaling boosts the therapeutic efficacy of anthracyclines upon increased type I interferon (IFN) secretion.\textsuperscript{217}

Pfrschke and co-authors (from Massachusetts General Hospital Research Institute and Harvard Medical School, Boston, MA, USA) found that autochthonous tumors lacking tumor-infiltrating lymphocytes (TILs) can be sensitized to immunological rejection via a Toll-like receptor 4 (TLR4)-dependent mechanism when suitable ICD inducers like oxaliplatin or cyclophosphamide are combined with ICBs targeting programmed cell death 1 (PDCD1; best known as PD-1)\textsuperscript{218} and/or cytotoxic T-lymphocyte associated protein 4 (CTLA4).\textsuperscript{218} Similarly, Stewart and colleagues (from MedImmune Ltd, Cambridge, United Kingdom) characterized a novel antibody directed against CD274 (best known as PD-L1) (MEDI4736), specifically engineered to prevent antibody-dependent cell-mediated cytotoxicity (ADCC),\textsuperscript{219} which exhibits potent antitumor activity when combined with oxaliplatin (only in immunocompetent mice).\textsuperscript{219} In a multi-modal combinatorial study, Rios-Doria and collaborators (from MedImmune, Gaithersburg, MD, USA) documented that liposomal doxorubicin induces potent ICD-associated innate and adaptive immune responses, in vivo, and effectively controls tumor growth, especially in combination with ICBs against CTLA4, PD-1, or its main ligand PD-L1 or with immunostimulatory fusion proteins targeting TNF receptor superfamily, member 4 (TNFRSF4; best known as OX40) or TNF receptor superfamily member 18 (TNFRSF18; best known as GITR).\textsuperscript{220} In a slightly different approach, Evans et al. (from Vaccinex, Inc., Rochester, NY, USA) reported that semaphorin 4D (SEMA4D) expression at the tumor invasive margin facilitates pro-tumorigenic inflammation, and that combining anti-SEMA4D monoclonal antibodies (mAbs) with ICBs or cyclophosphamide promotes immunological rejection in murine colorectal carcinoma models.\textsuperscript{221} Finally, Blake et al. (from QIMR Berghofer Medical Research Institute, Herston, Australia) observed that inhibiting CD96 – a negative regulator of natural killer (NK) cell activity\textsuperscript{212} – with ICBs or doxorubicin exerts superior antimetastatic effects in the lung.\textsuperscript{223}

Musahl et al. (from Max Planck Institute for Molecular Genetics, Berlin, Germany) observed that the long non-coding RNA, ncRNA-RB1, positively regulates overall CALR expression levels so that, following anthracyclines-induced ICD, ncRNA-RB1 supports surface-exposed CALR driven phagocytosis.\textsuperscript{224} In a similar fashion, Colangelo and co-authors (from University of Sannio, Benevento, Italy) found that the microRNA miR-27a specifically suppresses the exposure of CALR by mitoxantrone and oxaliplatin in colorectal cancer cells by negatively affecting the intracellular CALR trafficking pathway.\textsuperscript{225} In a study exploring novel combinatorial regimens, Lu and colleagues (from University of Alabama, Birmingham, AL, USA) documented that combining an inhibitor of thrombospondin 1 (THBS1; also known as TSP1) and transforming growth factor beta 1 (TGFβ1) signaling, i.e., SR131277, with bortezomib mediates superior anti-neoplastic effects as compared to either agents alone.\textsuperscript{226} In a different combinatorial approach, Hsu and colleagues (from National Yang-Ming University, Taipei, Taiwan) revealed that pre-conditioning with low-dose doxorubicin or paclitaxel before adoptive cell transfer (ACT) significantly improves antitumor immunity upon inhibition of NF-κB-regulated immunosuppressive factors.\textsuperscript{227} Similarly, Koo and collaborators (from Catholic University of Korea, Bucheon, Republic of Korea) found that X-shaped double-stranded oligodeoxynucleotide molecules (so-called “X-DNA”) that bind TLR9\textsuperscript{228} greatly enhance the antitumor efficacy of doxorubicin against colitis-associated colorectal carcinoma, via a mechanism that depends on DCs and T cells.\textsuperscript{229} Finally, Monk et al. (from University of Arizona College of Medicine-Phoenix, Phoenix, AZ, USA) documented that combining pegylated liposomal doxorubicin with a TLR8 agonist, i.e., motolimod, retards the growth of patient-derived ovarian carcinoma cells implanted in humanized mice.\textsuperscript{230}

In a study exploring resistance mechanisms, Buondonno et al. (from University of Torino, Torino, Italy) documented that treating osteosarcoma cells that overexpress ATP binding cassette subfamily B member 1 (ABCB1) – a plasma membrane transporter that actively extrudes several chemotherapeutics including doxorubicin – with mitochondria-targeted
doxorubicin overcomes chemoresistance to restore ICD and associated immune responses \textit{in vivo}.\textsuperscript{234} Finally, Shalapour and co-authors (from University of California San Diego, San Diego, CA, USA) reported that B cells producing class A immunoglobulins (IgAs), IL10 and PD-L1 in a TGFB1-dependent fashion act as immunsuppressive plasmocytes and prevent oxalatin-driven tumor rejection in different murine models of prostate cancer.\textsuperscript{232} Thounaojiam and colleagues (from Meharry Medical College, Nashville, TN, USA) found that bortezomib suppresses the growth of various solid tumors by upregulating the expression of various components of the Notch signaling pathway in lymphoid tissues including CD8⁺ CTLs, resulting in increased cytotoxic functions.\textsuperscript{219,233} Guillerey and collaborators (from QIMR Berghofer Medical Research Institute, Herston, Australia) observed that anti-myeloma immune response elicited by bortezomib or cyclophosphamide critically relies on CD226, which is crucial for the effector and cytotoxic functions of NK and CD8⁺ T cells.\textsuperscript{234} Finally, Wong and collaborators (from National University of Singapore, Singapore) screened a library of chemotherapeutically active platinum derivatives and characterized a Pt(II) N-heterocyclic carbene complex as a putative inducer of ICD (pending \textit{in vivo} validation), based on its capacity to trigger oxidative ER stress, CALR exposure, ATP secretion, and HMGBl release.\textsuperscript{235}

Taken together, these findings exemplify the attention currently focused around the molecular and cellular mechanisms through which the death of cancer cells responding to some (but not all) chemotherapeutics initiates a therapeutically relevant tumor-specific immune response.

**Completed clinical trials**

Since the publication of the latest Trial Watch dealing with this topic (April 2015), multiple peer-reviewed articles documented the outcome of clinical trials evaluating the efficacy of \textit{bona fide} ICD-inducing chemotherapeutics (\textit{i.e.}, doxorubicin, epirubicin, idarubicin, mitoxantrone, bortezomib, bleomycin, cyclophosphamide or oxaliplatin) along with ICD-associated immunological biomarkers.\textsuperscript{236,237} These publications were acquired from PubMed (http://www.ncbi.nlm.nih.gov/pubmed), and the initial list was manually curated to ensure relevance for this Trial Watch.

Several clinical studies demonstrated the beneficial immunostimulatory effects of ICD induction by chemotherapy. Loi \textit{et al.} (from Peter MacCallum Cancer Centre, Melbourne, Australia) documented that increased tumor infiltration by T cells is associated with improved prognosis in patients affected by triple-negative breast cancer (TNBC) with residual disease following neoadjuvant chemotherapy (consisting of doxorubicin, cyclophosphamide, and in some cases paclitaxel).\textsuperscript{238} Cornelissen and collaborators (from Erasmus MC Cancer Institute, Rotterdam, Netherlands) reported that combining DC-based vaccines with metronomic cyclophosphamide (which efficiently reduces circulating T\textsubscript{REG} cells) resulted in radiographic tumor control (and increased overall survival) in 8 out of 10 patients with malignant pleural mesothelioma patients.\textsuperscript{239} Schijns and colleagues (from Wageningen University, Wageningen, Netherlands) found that individuals with recurrent glioblastoma multiforme (GBM) receiving a vaccine composed of autologous antigens in combination with cyclophosphamide, experienced improved overall survival.\textsuperscript{240} Klein and co-authors (from Ludwig Institute for Cancer Research, Heidelberg, Australia) reported that cyclophosphamide promoted TAA-specific CD4⁺ T cell responses driven by a peptide-based vaccine targeting cancer/testis antigen 1B (CTAG1B; best known as NY-ESO-1)\textsuperscript{241} in patients with advanced melanoma, in the absence of T\textsubscript{REG} cell depletion.\textsuperscript{238} Murahashi \textit{et al.} (from Kyushu University, Fukuoka, Japan) evaluated a multipeptide-based vaccine combined with escalating doses of cyclophosphamide in patients with locally advanced, metastatic and/or recurrent gastrointestinal, lung or cervical cancer, achieving increased overall survival accompanied by increased TAA-specific T cells and peripheral T\textsubscript{REG} cell depletion.\textsuperscript{242} Tanis and collaborators (from The Netherlands Cancer Institute, Amsterdam, Netherlands) found that patients with liver metastases from colorectal carcinoma obtain a survival benefit from the FOLFOX therapeutic regimen (consisting of folinic acid, fluorouracil and oxaliplatin), which is accompanied by increased amount of CTLs at invasive tumor margin, as well as with mast cell infiltration.\textsuperscript{243} Collectively, these reports demonstrate that ICD-inducing chemotherapeutic regimens can prolong the survival of some cancer patients, and this often correlates with biomarkers of ongoing antigenicity.

Alongside, a few articles documented negative immunological consequences of chemotherapeutic regimens (at least potentially) triggering ICD, such as the R-CHOP regimen (consisting of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone). Specifically, Ito \textit{et al.} (from Fujita Health University Hospital, Aichi, Japan) found that diffuse large B-cell lymphoma (DLBCL) patients receiving R-CHOP experienced immunosuppression for two or more years following treatment, mainly manifesting with marked decrease in circulating CD20⁺ B cells and CD4⁺ T cells.\textsuperscript{244} Brown and collaborators (from University of Oxford, John Radcliffe Hospital, Oxford, UK) reported that patients with DLBCL receiving R-CHOP exhibited decreased levels of major histocompatibility complex, class II, DR alpha (HLA-DRA) on DLBCL cells, correlating with inferior overall survival.\textsuperscript{245} On a relatively conciliatory note though, Wu and co-authors (from The Second Hospital of Lanzhou University, Lanzhou, China) found that the low circulating levels of monocytes and monocytic myeloid-derived suppressor cells (MDSCs) have profound positive prognostic impact on the overall survival of DLBCL patients treated with R-CHOP.\textsuperscript{246} It is tempting to speculate, yet remains to be formally investigated, that the immunosuppressive effects of R-CHOP originate from prednisone.\textsuperscript{247} It would therefore be interesting to see whether an “R-CHO” regimen deprived of prednisone might exert superior immunostimulatory and therapeutic effects.

**Ongoing clinical trials**

Official sources list no less than 58 clinical trials evaluating the clinical efficacy of \textit{bona fide} ICD-inducing chemotherapeutic regimens in conjunction with relevant immune biomarkers.
initiated after April 2015 (since the publication of the latest Trial Watch dealing with this topic) (Fig. 1, Tables 1 and 2). These studies were retrieved from the ClinicalTrials.gov database (http://www.clinicaltrials.gov/), and the initial list was manually curated to ensure relevance for this Trial Watch.

Our survey revealed that multiple ICD-relevant biomarkers are being investigated as immunological outcomes across recently initiated clinical trials based on ICD-inducing chemotherapeutic regimens (Fig. 1A). These immunological biomarkers include: (1) parameters of broad T cell immunophenotyping, such as the so-called “Immuno-score” and the abundance of tumor-infiltrating or circulating CD3+ , CD4+ or CD8+ T cells, amongst others;251-253 (2) the circulating levels of multiple cytokines, such as interferon, gamma (IFNG; best known as IFN-γ);254 IL6,255 and tumor necrosis factor (TNF);256-258 (3) the amounts of TAA-specific T cells (via tetramer assays);259,260 (4) the tumor mutational load and/or the abundance of predicted neo-antigens (via genome or whole-exome sequencing);115,138,261-265 and (5) the levels of circulating biomarkers of ICD (via proteomic or metabolomic assays).49,266 Alongside, several immunological biomarkers with indirect relevance for ICD and its therapeutic outcomes are also being tested, including the abundance of circulating or tumor-infiltrating NK cells, MDSCs and TREG cells, as well as the expression levels and activation status of immune checkpoints.4,261,267-273 In this setting, the profiling of TREG cells is being performed in most cases to estimate the ability of cyclophosphamide to specifically target these immunosuppressive cells.274-276 It will be interesting to see whether the peripheral profile (in terms of abundance and activation status) of CD4+ and/or CD8+ T cells can serve as reliable biomarker for the immunological consequences of chemotherapy.4,277 Preclinical and (mostly retrospective) clinical data advocate that tumor-infiltrating T cells provide superior prognostic and/or predictive information,251,267,278 but the identification of a reliable circulating biomarker would be a major asset for immunomonitoring.

A majority of the clinical trials surveyed here aim at testing cyclophosphamide, epirubicin, doxorubicin or oxaliplatin, typically as on-label therapeutic interventions and often as part of gold standard chemotherapeutic regimen (Fig. 1B, Tables 1 and 2). In some cases, the ICD-inducing potential of the chemotherapeutic regimen of choice was cited as part of the rational to the study. However, we were unable to identify any clinical trial specifically comparing the ICD-inducing potential of these agents with a relevant non-immunogenic chemotherapeutic. In a majority of cases, cyclophosphamide is administered with the primary aim of inhibiting or depleting (systemic) TREG cells. However, considering the wide array of immunological biomarkers monitored in these clinical trials (Fig. 1A), the ICD-inducing potential of cyclophosphamide may also become apparent.

**Figure 1.** Current clinical trials testing immunogenic cell death (ICD)-inducing chemotherapies in oncological indications. A. Distribution by immunological biomarker (biomarkers directly relevant for ICD are in bold). B. Distribution by main chemotherapeutic agent. C. Distribution by oncological indication. D. Number of clinical trials currently testing ICD-inducing chemotherapeutic regimens in combination with immunotherapy. CIK, cytokine-induced killer; CSF1, colony stimulating factor 1; DC, dendritic cell; mAb, monoclonal antibody; MDSC, myeloid-derived suppressor cell; NK, natural killer; TAA, tumor-associated antigen; TLR, Toll-like receptor; TREG regulatory T.
As for oncological indication, the studies we retrieved are currently enrolling patients with breast carcinoma (16 trials), lung cancer (in particular non-small cell lung carcinoma, NSCLC) (5 trials), colorectal carcinoma (3 trials), prostate cancer (3 trials), and other cancer types (31 trials) (Fig. 1C, Table 1 and 2). In general, solid tumors (51 trials) are preferred to hematological malignancies (8 trials). Possibly, this reflects, (1) the high sensitivity of several hematological tumors to standard-of-care treatments, and/or (2) the fact that hematological malignancies often stem from components of the immune system, resulting in compromised immune functions and limited susceptibility to multiple forms of immunotherapy and immunologic chemotherapies.\(^{279,280}\) In multiple clinical studies, bona fide ICD-inducing chemotherapeutic regimens are combined with agents that elicit ICD per se, such as radiation therapy (2 trials), or considerably boost the immunogenicity of cancer cells, such as taxanes or zoledronic acid (10 trials). Finally, in a limited amount of trials, ICD inducers are combined with targeted anticancer agents, including the inhibitor of JAK kinases ruxolitinib (1 trial) and the inhibitor of mechanistic target of rapamycin kinase (MTOR) rapamycin\(^{281,282}\) (also known as sirolimus, or its derivative everolimus) (2 trials) (Tables 1 and 2).

Fitting well within the current oncology landscape, a majority of clinical trials surveyed for this Trial Watch combine ICD-inducing chemotherapeutic regimens with bona fide immunotherapies (Fig. 1D, Tables 1 and 2). These include: (1) immunostimulatory cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), IL2, IFN-α and IRX-2, a cell-free mixture of cytokines encompassing IL1, IL2, IL6, IL8, IL10, IL12, TNF, IFN-γ and colony stimulating factor 1 (CSF1, also called macrophage colony stimulating factor 1 or M-CSF); (2) ICBS such as the PD-1-targeting agents pembrolizumab\(^{283}\) and SHR-1210,\(^{284}\) and the PD-L1-targeting agents avelumab\(^{285}\) (3) the inhibitors of indoleamine 2,3-dioxygenase 1 (IDO1);\(^{138,285}\) (4) adoptively transferred T cells, often CD8+ T cells or PD-1-de\(^\text{+}\) T cells;\(^{286}\) (5) tumor-targeting mAbs, such as the CD20-targeting rituximab,\(^{287}\) the vascular endothelial growth factor (VEGF)-targeting mAb bevacizumab,\(^{288}\) and various molecules targeting HER2,\(^{289}\) (6) anticancer vaccines,\(^{12,53,290,291}\) and (7) oncolytic viruses\(^{292,293}\) (Fig. 1D, Table 1 and 2).

Finally, most of the clinical trials we surveyed are ongoing (i.e., either actively recruiting or initiated/registered but not yet recruiting), with a few notable exceptions. NCT03050814 (a Phase 2 study testing oxaliplatin-based chemotherapy in combination with PD-L1 blockade or bevacizumab in colorectal carcinoma patients) has been suspended pending further discussion with the US FDA, because an unspecified number of individuals died within 30 days of treatment. On a different note, NCT02419170 (a Phase I study testing DC-based vaccination plus cyclophosphamide in NSCLC patients) has been withdrawn prior to enrollment because the investigator manufacturing the vaccines left the concerned institution. Finally, NCT02461121 (a Phase III trial comparing cyclophosphamide-containing versus cyclophosphamide-free preconditioning in patients with acute myeloid leukemia allocated to transplantation) and NCT02655458 (a Phase I study involving cyclophosphamide-containing preconditioning in multiple

### Table 1. Current clinical trials evaluating the therapeutic and immunological profile of ICD-inducing chemotherapeutic regimens.\(^*\)

| Drug | Indication(s) | Phase | Status | Notes | Ref. |
|------|---------------|-------|--------|-------|------|
| Bleomycin | Rectal cancer | II | Recruiting | As single agent in the context of electro-chemotherapy | NCT03040180 |
| Bortezomib | Lung cancer | I | Not yet recruiting | Combined with a DRibble vaccine and DC/CIK therapy | NCT03057340 |
| Doxorubicin | Breast carcinoma | I/II | Recruiting | Combined with epirubicin, cyclophosphamide, fluorouracil or methotrexate | NCT02897700 |
| | | | Recruiting | Combined with debatine, cyclophosphamide and paclitaxel | NCT02957968 |
| | | | Recruiting | Combined with erubulin and cyclophosphamide | NCT02623972 |
| | | | Recruiting | Combined with cyclophosphamide, ruxolitinib and paclitaxel | NCT02876302 |
| Epirubicin | Pediatric solid tumors | I | Recruiting | As lypo-thermosensitive liposomal doxorubicin | NCT02536183 |
| | | | Recruiting | Combined with doxorubicin, cyclophosphamide, fluorouracil or methotrexate | NCT02897700 |
| | | | Recruiting | Combined with paclitaxel, cyclophosphamide and MEDI4736 | NCT02685059 |
| | | | Recruiting | Combined with docetaxel, cyclophosphamide/ capcitabine or fluorouracil/cyclophosphamide | NCT02897050 |
| | | | Recruiting | Combined with cyclophosphamide, fluorouracil, trastuzumab or pertuzumab | NCT03144947 |
| Idrubicin + mitoxantrone | Acute promyelocytic leukemia | III | Recruiting | Combined with arsenic trioxide, tretinoin, cytarabine, mercaptopurine and methotrexate | NCT02688140 |
| Oxaliplatin | Colorectal carcinoma | n.s. | Recruiting | Combined with 5-fluorouracil and bevacizumab | NCT02817718 |
| | | | Recruiting | Combined with trifuridine, bevacizumab and nivolumab | NCT02846443 |
| | | | Recruiting | Combined with 5-fluorouracil, leucovorin, bevacizumab, capcitabine, Ad-CEA vaccine and avelumab | NCT03050814 |
| Gastrointestinal cancer | IV | Not recruiting | Combined with cinobufotalin | NCT02860429 |
| Gastric cancer | II | Recruiting | Combined with capcitabine, epirubicin and pembrolizumab | NCT02918162 |
| Pancreatic cancer | I | Recruiting | Combined with folic acid, rituximab, 5-fluorouracil, paclitaxel, gemcitabine and a DC-based vaccine | NCT02548169 |

**Abbreviations:** Ad-CEA, adenovirus vector encoding carcinoembryonic antigen; CIK, cytokine-inducer killer; DC, dendritic cell; n.s., not specified; *Initiated between 1st April 2015 and 30th June 2017.*
myeloma patients) have been completed. However, to the best of our knowledge, the results of these studies have not been released yet.

Concluding remarks

A number of ICD-inducing chemotherapeutic regimens are currently approved by the US FDA or equivalent regulatory agencies worldwide for use in cancer patients. However, the widespread use of these treatments has been implemented mostly on empirical (rather than immunological) grounds.\(^{300,296–300}\) Indeed, the possibility that chemotherapy and other forms of treatment (including radiotherapy and photodynamic therapy) might promote an immunogenic form of cancer cell death has been overlooked for several decades.\(^{301–303}\) Thus, current anticancer drugs have been developed in immunodeficient preclinical models and in clinical trials devoid of any form of immunomonitoring, mainly most often aimed at identifying maximum tolerated doses (MTDs).\(^{298,304}\) Nonetheless, a majority of currently available anticancer agents mediate on-target or off-target immunostimulatory effects, which strongly argues against an irrelevant role for the immune system in the therapeutic effects of these treatments.\(^{305–308}\)

Moreover, in various cases, chemotherapeutics applied through multiple treatment cycles may negatively affect the immune system, by causing lymphopenia or leukopenia, thereby further compromising antitumor immune responses.\(^{44,53,34}\) One of the major challenges for the future will be to identify doses and administration schedules that mediate maximal immunostimulatory effects.\(^{297,309,310}\) Accumulating evidence suggest indeed that metronomic chemotherapy and hypofractionated radiation (rather than chemotherapy at the MTD and high single-dose radiation) exerts superior immunostimulatory (and hence therapeutic, at least in some settings) effects.\(^{311–314}\) Alongside, it will be important to devise highly efficient combinatorial regimens that harness not only the ability of some treatments to drive ICD, but also the off-target immunostimulatory effects of a variety of agents. We are convinced that conventional therapeutic regimens – if properly employed – are a very powerful and relatively economical tool to drive clinically relevant antitumor immune responses.

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Table 2. Current clinical trials evaluating the therapeutic and immunological profile of cyclophosphamide-based chemotherapy.\(^{2}\)

| Indication(s)                  | Phase | Status     | Notes                                                                 | Ref.          |
|-------------------------------|-------|------------|----------------------------------------------------------------------|---------------|
| Acute lymphoblastic leukemia  | II    | Recruiting | Combined with standard-of-care chemotherapy                         | NCT02823558   |
| Acute myeloid leukemia        | III   | Completed  | Combined with standard-of-care chemotherapy and anti-lymphocyte globulin | NCT02461121   |
| B-cell lymphoma               | I/I   | Recruiting | Combined with fludarabine and CD19-specific CAR-T cells              | NCT03146533   |
| Breast carcinoma              | I     | Unknown    | Combined with radiotherapy                                           | NCT03441270   |
|                               | II    | Recruiting | Combined with indomethacin, omeprazole, dietary supplements (e.g.,  | NCT02950259   |
|                               |       |            | multivitamins) and IRX-2                                             |               |
| Chronic lymphocytic leukemia  | II    | Not recruiting | Combined with peptide vaccination                                      | NCT03012100   |
| DLBCL                         | I     | Recruiting | Combined with fludarabine and CD19-specific CAR-T cells              | NCT02431988   |
| EBV-associated malignancies\(^{2*}\)  | I/I | Recruiting | Combined with fludarabine and PDCD1-deficient EBV-specific CTLs | NCT03044743   |
| Esophageal cancer             | I     | Recruiting | Combined with PDCD1-deficient CTLs and IL2                          | NCT03081715   |
| Gastrointestinal cancer       | I     | Not recruiting | Combined with ACT, IL2 and pembrolizumab                              | NCT02757391   |
| Gynecological tumors          | II    | Recruiting | Combined with epacadostat and DPX-Survivac                          | NCT02785250   |
| Gastrointestinal cancer       | II    | Recruiting | Combined with pembrolizumab and bevacizumab                         | NCT02853318   |
| Hematological malignancies\(^{2*}\)  | I/I | Not recruiting | Combined with a DC-based vaccine and GM-CSF | NCT02790993   |
| Hepatocellular carcinoma      | I/I   | Not recruiting | Combined with IMA970 A plus C8102                                    | NCT03203005   |
| Lung cancer                   | I     | Withdrawn  | Combined with DC-based vaccine                                       | NCT02419170   |
| Melanoma                      | I/I   | Recruiting | Combined with polyICLC and MHP vaccine                               | NCT02425306   |
| Mesothelioma                  | I/I   | Recruiting | Combined with pembrolizumab and ONCOS-102                            | NCT03003676   |
| Multiple myeloma              | I     | Completed  | Combined with lenalidomide and elotuzumab                            | NCT02655458   |
| Neuroendocrine tumors         | I     | Recruiting | Combined with IFN-\(\gamma\)                                        | NCT02838342   |
| Osteosarcoma                  | I     | Recruiting | Combined with sirolimus, methotrexate and zoledronic acid           | NCT02517918   |
| Prostate cancer               | I     | Recruiting | Combined with DNA-based vaccine                                       | NCT02390063   |
| Renal cell carcinoma          | I     | Not recruiting | Combined with PDCD1-deficient CTLs and IL2                       | NCT02867345   |
| Sarcoma                       | II    | Recruiting | Combined with pembrolizumab                                          | NCT02867332   |
| Solid tumors                  | I/I   | Not recruiting | Combined with a TLR8 agonist and pegfilgrastim                      | NCT02606536   |
|                               |       | Recruiting | Combined with a DC-based vaccine and GM-CSF                         | NCT02705703   |
|                               |       | Recruiting | Combined with fludarabine, neoaigntin-specific T cells, PDCD1 inhibition and IL2 | NCT03171220   |

Abbreviations: ACT, adoptive cell transfer; CAR, chimeric antigen receptor; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DLBCL, diffuse large B-cell lymphoma; DPX, DepoVax\(^{2*}\); EBV, Epstein-Barr virus; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; MHP, melanoma helper peptides; polyICLC, polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose; TAA, tumor-associated antigen; TLR, Toll-like receptor \(^{1*}\)Initiated between 1\(^{1*}\) April 2015 and 30\(^{1*}\) June 2017 \(^{2*}\)Including gastric carcinoma, nasopharyngeal carcinoma, T-cell lymphoma, Hodgkin lymphoma and DLBCL.
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Abbreviations

ACT: adoptive cell transfer
DAMP: damage-associated molecular pattern
DC: dendritic cell
ER: endoplasmic reticulum
FDA: Food and Drug Administration
GM-CSF: granulocyte-macrophage colony-stimulating factor
ICB: immune checkpoint blocker
ICD: immunogenic cell death
IFN: interferon
IL: interleukin
mAb: monoclonal antibody
MTD: maximum tolerated dose
NSCLC: non-small cell lung carcinoma
RCD: regulated cell death
TAA: tumor-associated antigen
TIL: tumor-infiltrating lymphocyte
TLR: Toll-like receptor

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