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INVITED REVIEW

Immunogenic cell death (ICD) enhancers—Drugs that enhance the perception of ICD by dendritic cells

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Summary

The search for immunostimulatory drugs applicable to cancer immunotherapy may profit from target-agnostic methods in which agents are screened for their functional impact on immune cells cultured in vitro without any preconceived idea on their mode of action. We have built a synthetic mini-immune system in which stressed and dying cancer cells (derived from standardized cell lines) are confronted with dendritic cells (DCs, derived from immortalized precursors) and CD8⁺ T-cell hybridoma cells expressing a defined T-cell receptor. Using this system, we can identify three types of immunostimulatory drugs: (i) pharmacological agents that stimulate immunogenic cell death (ICD) of malignant cells; (ii) drugs that act on DCs to enhance their response to ICD; and (iii) drugs that act on T cells to increase their effector function. Here, we focus on strategies to develop drugs that enhance the perception of ICD by DCs and to which we refer as “ICD enhancers.” We discuss examples of ICD enhancers, including ligands of pattern recognition receptors (exemplified by TLR3 ligands that correct the deficient function of DCs lacking FPR1) and immunometabolic modifiers (exemplified by hexokinase-2 inhibitors), as well as methods for target deconvolution applicable to the mechanistic characterization of ICD enhancers.

KEYWORDS

apoptosis/autophagy, cancer, dendritic cells, signal transduction, T cells, tumor immunity

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1 | INTRODUCTION

Immunostimulatory drugs are nowadays routinely used for the (immuno) therapy of a wide range of cancer types. Indeed, conventional antineoplastics including cytotoxic chemotherapeutics and so-called targeted agents only act on a minor fraction of cancer types usually leading to the development of acquired resistance due to the unprecedented plasticity of malignant cells.^{1,2} In sharp contrast, immunotherapies targeting immune checkpoints (in particular the interaction between programmed cell death-1, PD-1, and its ligand, PD-L1, as well as cytotoxic T lymphocyte antigen-4, CTLA-4) can be widely used through the oncological spectrum, with a marked tendency to move from adjuvant settings (immunotherapy after surgery) to neoadjuvant treatments (immunotherapy before surgery).^{3,4} Thus, interventions on the ecosystem composed by malignant and immune cells, as well as by other cell types, are more efficient against neoplasia than drugs that directly act on cancer cells.^{5,6}

Paradoxically, it appears that even before immune checkpoint inhibitors (ICIs) targeting CTLA-4, PD-1, or PD-L1 were approved for clinical use as “immunotherapies”, successful anticancer therapies were de facto acting as immunotherapies.⁷ Indeed, many cytotoxic and targeted anticancer agents stress and kill malignant cells in a way that they become recognizable to the immune system.^{8,9} In mouse models, such agents—that we dubbed “immunogenic cell death (ICD) inducers”—lose their anticancer effects upon depletion of T cells or dendritic cells (DCs).^{10–12} Similarly, in patients, the therapeutic efficacy of ICD inducers correlates with the infiltration of tumors by activated DCs and cytotoxic T lymphocytes, strongly suggesting that the anticancer immune response determines treatment outcome.^{13–15} Several major classes of anticancer drugs have been discovered to induce ICD, as described for anthracyclines,¹⁰ taxanes,¹³ some platinum-based compounds such as oxaliplatin,¹⁶ ionizing irradiation,^{17,18} photodynamic therapy,^{19,20} as well as specific tyrosine kinase inhibitors^{21,22} and oncolytic viruses.^{23,24} As an additional hint in favor of the idea that such agents mediate their effects through the immune system, it appears that combinations of ICD inducers with ICIs are particularly efficient at the clinical level.^{25,26}

Schematically, the immunogenicity conferred by ICD requires the combination of two major features, (i) antigenicity and (ii) adjuvanticity.²⁷ With respect to antigenicity, cancer cells differ from their normal counterparts in their antigenic makeup including the immunopeptidome, that is, the sum of antigenic peptides bound to MHC Class I molecules on the surface.²⁸ Cancer cells usually express a set of peculiar antigens that may arise from mutations (“neo-antigens”) or from stress response resulting into alterations of the transcriptome including that of noncoding RNAs yielding cryptic transcripts (“non-neo-antigens” that often are autoantigens). Moreover, anticancer drugs may induce genomic mutations and modulate the non-mutated transcriptome, hence enhancing the antigenicity of malignant cells.²⁹

Adjuvanticity results from alterations of the cell surface (for instance due to the exposure of calreticulin, CALR, and other

chaperones or due to alterations in the glycocalyx),^{17,30} as well as from the release of other “danger associated molecular patterns” (DAMPs) from stressed or dying cells into the extracellular space. DAMPs are a compendium of proteins and metabolites that are usually sequestered within cells (such as adenosine triphosphate, ATP; annexin A1, ANXA1; and high mobility group B1, HMGB1) or are usually not expressed by non-stressed cells, requiring their transcriptional activation (such as Type-1 interferons).^{31–34}

The changes affecting the cell surface of cancer cells undergoing ICD, as well as the local secretome surrounding dying cancer cells, facilitate the immune recognition of cancer cells via a multipronged effect on DCs. Thus, DC precursors are attracted into the tumor bed (by chemotaxis depending on ATP that acts on purinergic P2Y2 receptors expressed by myeloid cells),³⁴ lured into the vicinity of dying cancer cells (due to the effect of ANXA1 on formyl peptide receptor-1, FPR1),³³ stimulated to engulf portions of the tumor cells (due to exposure of CALR that acts as a DC-specific “eat me” signal),¹⁷ and caused to mature (due to the effect of HMGB1 on Toll-like receptor-4, TLR4).³¹ As an end result, fully differentiated and mature DCs present tumor-associated antigens to T cells, hence initiating a cellular immune response against cancer cells. This immune response involves the action of CD8⁺ cytotoxic T lymphocytes specific for tumor-associated antigens. Unfortunately, tumor cell lysis by such CD8⁺ T cells often occurs in a transient fashion due to T-cell exhaustion.³⁵

Over the past years, we have not only identified (some of) the rules dictating the tripartite interaction of (i) cancer cells undergoing ICD, (ii) DCs, and (iii) T cells to understand the mode of action of successful anticancer drugs, but we also developed a variety of screening systems to identify new drugs (or to repurpose old drugs) that induce ICD in malignant cells^{36,37} or enhance the immune response by acting on DCs and T cells.^{38,39} Here, we will briefly review the design of such screening systems, which so far has led to the identification of two novel anticancer drugs cataloged as “ICD inducers” and that received approval by the US Food and Drug Administration (FDA) as well as by the European Medicines Agency (EMA).^{40–43} Such drugs were found by screening compound libraries for their capacity to induce the exposure or release of DAMPs from human cancer cell lines *in vitro*, followed by *in vivo* experiments (in mice) and clinical trials proving their immunostimulatory effects.^{36,37} However, we believe that alternative screening methods that involve immune cells (DCs or T cells) may be at least as efficient in identifying new immunostimulatory drugs.

In this review, we will briefly touch upon the development of screening methods for identifying (i) ICD inducers (that act on cancer cells), (ii) T-cell stimulators (that directly act on T lymphocytes) and (iii), most importantly, drugs that improve the perception of ICD by DCs and that we refer to as “ICD enhancers.” For this latter category of agents, we will delineate methods for target deconvolution before we explore the perspectives of this field of immunopharmacological research.

2 | SCREENING FOR ICD INDUCERS

Regulated cell death (RCD) occurs in distinct forms (e.g., apoptosis, cuproptosis, ferroptosis, necroptosis, parthanatos, and pyroptosis), that differ in their morphological appearance, biochemical pathways, and functional outcome.⁴⁴ However, it would be a bold oversimplification to consider that these RCD subroutines are intrinsically endowed with more or less immunogenicity.⁹ Rather, the history of cell stress responses that have been induced before the activation of lethal signaling pathways plays a major role in determining whether cell death ignites an immune response against dead-cell antigens.

Apoptosis occurring in the context of tissue homeostasis is usually non-immunogenic, hence leading to the removal of dead cells by macrophages, a process called efferocytosis that by definition is silent (i.e., does not initiate a local tissue reaction).⁴⁵ In strong contrast, apoptosis that is preceded by the so-called integrated stress response (ISR) consisting in the phosphorylation of eukaryotic translation initiation factor 2a (eIF2a) by a limited set of kinases (eIF2a alpha kinase 2, EIF2AK2, best known as protein kinase R, PKR; and EIF2AK3, best known as PKR-like endoplasmic reticulum kinase, PERK) tends to be immunogenic. Indeed, the ISR is required for two premortem stress responses, (i) the activation of macroautophagy, which as an end result facilitates the lysosomal secretion of the DC attractor ATP,^{46,47} and (ii) the exposure of CALR, which facilitates the uptake of dying cells by DCs rather than by macrophages.^{48,49} The ISR is often induced by chemotherapeutics that inhibit DNA-to-RNA translation,⁵⁰ as well as by microtubular toxins including taxanes and vinca-alkaloids.¹³ Another premortem stress response that is required for optimal immunogenicity is the secretion of Type-1 interferons by cancer cells.³² Moreover, in specific cases, the activation of the nuclear factor κ B (NF- κ B) pathway may contribute to immunogenic cell death.^{51,52}

The aforementioned caveat explains why it is not realistic to attempt the identification of ICD inducers by the mere assessment of cell death events. There is no cell death modality that would be intrinsically immunogenic. To identify ICD inducers, we rather opted for a cell death modality-agnostic approach. For this, we equipped human cancer cells (typically U2OS osteosarcoma cells) with biosensors for the measurement of premortem autophagy (which we assessed by monitoring the appearance of cytoplasmic puncta of green fluorescent protein [GFP] fused to microtubule-associated protein 1A/1B light chain 3B [LC3]),⁵³ the exposure of the DAMP CALR (that we measured by means of a CALR- red fluorescent protein [RFP]-fusion protein that distributes to the periphery of the cell)⁵⁴ or the release of the DAMP HMGB1 (that we detected as a HMGB1-GFP-fusion protein usually present in the nucleus, but released upon cell death).⁵⁵ Cancer cells equipped with these biosensors were incubated with individual compounds contained in publicly available chemical libraries or small compound collections provided by industrial partners (Table 1), and then the surrogate markers of ICD were systematically scored by means of fluorescence

videomicroscopy coupled to automated image analysis. In addition, DAMPs contained in the supernatants of cell cultures were quantified by different methods such as commercial luciferase-based assays to measure ATP and a biosensor cell responsive to Type-1 interferons⁵⁶ (Figure 1).

Using this approach, we have identified numerous candidate ICD inducers that were able to induce all the major stigmata of immunogenic cell stress and death in vitro on cultured cancer cells. In a subsequent round of validation experiments, candidate agents were tested on mouse cancer cells in vitro to confirm their broad capacity to elicit immunogenic stress and death beyond species barriers. If these in vitro results were deemed satisfactory, mouse cancer cells treated with the ICD inducer candidate drug in vitro were injected subcutaneously into syngeneic immunocompetent hosts into the right flank. Mice were subsequently (usually within 7–14 days) rechallenge with live cancer cells into the opposite flank and a reduction in tumor growth or—ideally—the complete absence of tumor development was interpreted as a sign of ICD that “vaccinates” against cancer (Figure 2A). Moreover, candidate drugs were evaluated for their capacity to control the growth of established cancers more efficiently in immunocompetent than in immunodeficient mice (usually athymic mice or mice injected with antibodies depleting CD4⁺ and CD8⁺ T cells) (Figure 2B) and to synergize with ICIs^{36,37} (Figure 2C). We only consider drugs that fulfill all these criteria, including the in vivo vaccination and immune-dependent therapeutic response, as bona fide ICD inducers. That said, there are efforts to combine distinct anticancer drugs, each of which alone do not induce ICD, among each other to obtain combinatorial ICD-inducing effects.⁵⁷ Moreover, novel nanomedicine-relevant procedures are allowing to generate novel galenic formulations for ICD induction that involve multiple compounds transcending the traditional limits of pharmacology.^{58–60} Our approach to identify ICD inducers by drug screening is only apt for the identification of single compounds.

In several cases, some kind of clinical validation could be obtained for ICD inducers (Figure 2D). For example, anthracycline- or taxane-induced effects against breast cancer are coupled to major changes in the immune infiltrate that predict clinical outcome.^{13,61} Oxaliplatin (which is an efficient ICD inducer) synergizes better with ICIs in patients with gastric or esophageal cancer than cisplatin (which is a poor ICD inducer).^{26,62,63} Moreover, the clinical activity of oxaliplatin administered into the hepatic artery for the treatment of colorectal cancer metastasis correlated with signs of DC activation.^{64,65} In patients with non-small cell lung cancer undergoing immunotherapy, elevations in plasma levels of CALR by a factor >2 over baseline correlate with improved progression-free and overall survival.⁶⁶ Increased eIF2a phosphorylation and plasma membrane exposure of CALR on malignant cells correlate with better survival in acute myeloid leukemia,^{67,68} non-small cell lung cancer,⁶⁸ as well as ovarian cancer.⁶⁹ The clinical use of cardiac glycosides, which induce ICD, during chemotherapy is coupled to improved overall survival in cohorts of breast, colorectal, head and neck, and hepatocellular carcinoma patients, especially when they were treated with non-ICD-inducing cytotoxicants (other than anthracyclines and oxaliplatin).⁷⁰

TABLE 1 Identification of immunogenic cell death (ICD) inducers and enhancers in various screening campaigns.

ICD inducer or enhancer	Evidence							Ref.
	Compound collection (number of molecules)	ICD hallmarks	Anticancer vaccination	T-cell dependence	ICI sensitization	Clinical translation		
Doxorubicin	N/A	x	x	x	x	x	10,111,112	
Mitoxantrone	N/A	x	x	x	x	x	112	
Oxaliplatin	N/A	x	x	x	x	x	16	
Digoxin	US drug library (~1200)	x	x	x	x	x	70	
Septacidin	NCI putative anticancer agents (~900)	x	x	x	x	x	113	
LTX-315	Selection of oncolytic agents	x	x	x	x	x	77	
LTX-401	Selection of oncolytic agents	x	x	x	x	x	114	
DTT-205, DTT-304	Selection of oncolytic agents	x	x	x	x	x	115	
Epigenetic modifiers (azacitidine, decitabine, SAHA)	Prestwick chemical library (~1200)	x	x	x	x	x	116	
ALK inhibitors (ceritinib, crizotinib)	Public chemogenomic set for protein kinases (~500)	x	x	x	x	x	21,22,117,118	
Thiostrepton	Prestwick chemical library (~1200) + flavonoids, fatty acids	x	x	x	x	x	119	
Picropodophyllin	Natural compound library (~65,000) + polyphenols (~1000)	x	x	x	x	x	120	
Isobavachalcone	Polyphenols (~1000)	x	x	x	x	x	121	
Dactinomycin	Virtual screening of ~50,000 compounds	x	x	x	x	x	50,122	
Belantamab mafodotin	Selection of anticancer agents	x	x	x	x	x	42	
Lurbinectedin	Selection of marine compounds	x	x	x	x	x	40,41	
Poly (I:C)	TLR agonist library	x	x	x	x	x	38	
TL-532	TLR3 agonist	x	x	x	x	x	98	
Astemizole, Ikarugamycin	ICCB library (~500), neuroendocrine factors (~700), Prestwick chemical library (~1200)	x	x	x	x	x	86	

Abbreviations: ALK, anaplastic lymphoma kinase; ICCB, Institute of Chemistry and Cell Biology; ICI, immune checkpoint inhibitor; N/A, not applicable; NCI, National Cancer Institute; poly (I:C), polyinosinic:polycytidylic acid; SAHA, suberoylanilide hydroxamic acid; TLR, Toll-like receptors; US, United States.

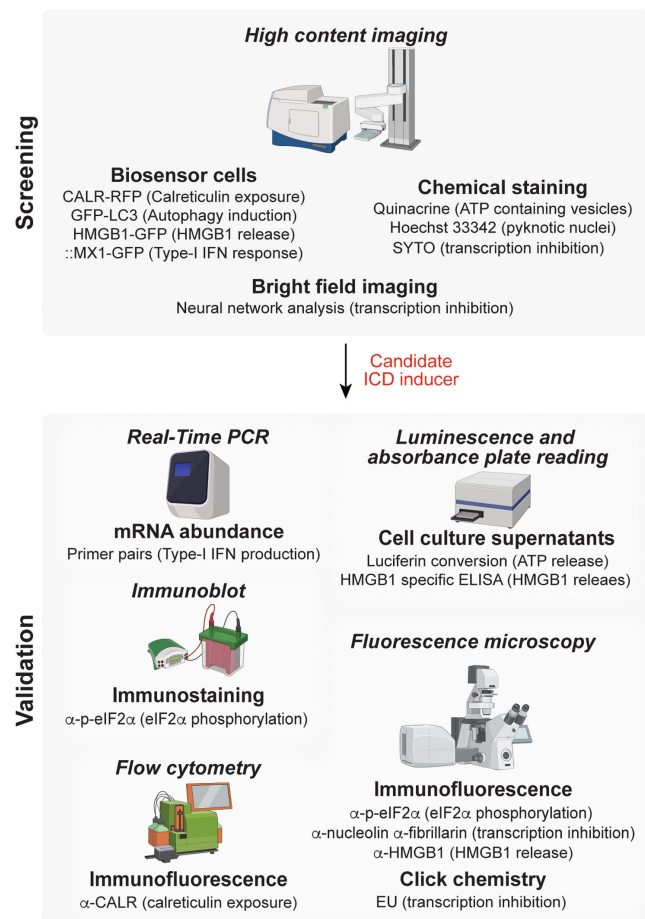


FIGURE 1 In vitro screening and functional validation of immunogenic cell death (ICD) inducers. Surrogate markers of ICD can be systematically screened in the form of fluorescent biosensor cells that express ICD-related danger-associated molecular patterns (DAMPs) coupled to green or red fluorescent proteins (GFP, RFP). Furthermore, chemical biosensors, click chemistry, immunofluorescence, and label-free microscopy can be employed to monitor characteristics of bona fide ICD including stalled DNA-to-RNA transcription. Altogether, image-based phenotypic drug screening coupled to automated image analysis can facilitate the high throughput screening of compound libraries for the identification of novel ICD-inducing agents. The exposure of calreticulin (CALR) on the plasma membrane can be assessed by immunofluorescence staining and flow cytometry. In addition, DAMPs contained in the supernatants of cell cultures can be quantified by ELISA (for proteins), luciferin conversion assays (for ATP), and biosensor cells responsive to Type-1 interferons. Immunoblots and real-time PCR on whole cell extracts can be used for further validating transcriptional adaptations and posttranslational modifications in response to ICD-related cell stress.

Obviously, the level of evidence of such retrospective, purely correlative clinical analyses is limited.

Fortunately, several agents that were characterized in our ICD inducer discovery platform underwent prospective clinical evaluation in interventional trials (Figure 2E). Importantly, a randomized, double-blind clinical trial combining ICD-inducing chemotherapy (pegylated liposomal doxorubicin plus cyclophosphamide)^{10,38,71} and the

PD-L1-blocking antibody atezolizumab have revealed clinical benefit against metastatic triple-negative breast cancer.⁷² Similarly, the combination of doxorubicin (without a specific galenic formulation) and PD-1 blockade with pembrolizumab is active against metastatic triple-negative breast cancer and exhibited changes in circulating T cells (an expansion of exhausted CD8⁺ T cells) that might be interpreted as a sign of a T-cell response.⁷³ An example of a new drug characterized on our platform is lurbinectedin, which was found to induce ICD in mouse experiments⁴⁰ and then was later successfully tested against relapsed small-cell lung cancer⁷⁴ leading to its FDA and EMA approval.⁴¹ Lurbinectedin monotherapy also shows encouraging activity against pretreated germline BRCA1/2 metastatic breast cancer.⁷⁵ Based on our preclinical results suggesting a positive interaction between lurbinectedin and ICIs,⁴⁰ multiple clinical studies have been launched to evaluate such combination effects in cancer patients. Another example is provided by belantamab mafodotin, a BCMA-targeting antibody-drug conjugate. In this case, the ICD-inducing effects were published by the manufacturer in a delayed fashion,⁴² after the FDA and EMA extended clinical approval for the use of drug in patients with refractory or relapsed multiple myeloma based on positive clinical results.⁷⁶ Nonetheless, thus far no clinical trials combining belantamab mafodotin with ICIs have been filed, perhaps due to the elevated toxicity of the drug. LTX-315, which is yet another ICD inducer discovered on our platform,^{77,78} is currently undergoing clinical evaluation (<https://beta.clinicaltrials.gov>). The platinum-based compound PT-112, which was found by other investigators to induce ICD,⁷⁹ has undergone exploratory evaluation in cancer patients⁸⁰ and is now entering more advanced clinical trials.

In conclusion, it appears that our current knowledge on ICD is sufficiently developed to identify clinically useful anticancer drugs through a continuum of in vitro screening, mouse experimentation, clinical correlation studies, and interventional trials. It remains to be seen whether the identification of additional ICD-related DAMPs that is ongoing, as exemplified for F-actin^{81,82} and mitochondrial transcription factor A, (TFAM)⁸³ will allow for a further refinement of ICD screens. At this point, we are still in the process of improving the in vitro pipeline leading to the discovery of novel ICD inducers. This involves the use of artificial intelligence (AI)-based algorithms to predict ICD-favorable physicochemical properties of small molecules, hence narrowing down the number of candidate compounds that should undergo in vitro screening on biosensor cell lines.⁸⁴ Another strategy for cost reduction consists in the replacement of fluorescence biosensor-based screens by label-free brightfield microscopy and image analyses by deep neural networks.⁸⁵ Finally, a better understanding of the proximal targets of ICD inducers might provide a rationale to simplify the current workflow of screening procedures.

3 | SCREENING FOR T-CELL STIMULATORS

We recently performed a screen in which we searched for pharmacological agents that stimulate interleukin-2 (IL2) production by B3Z

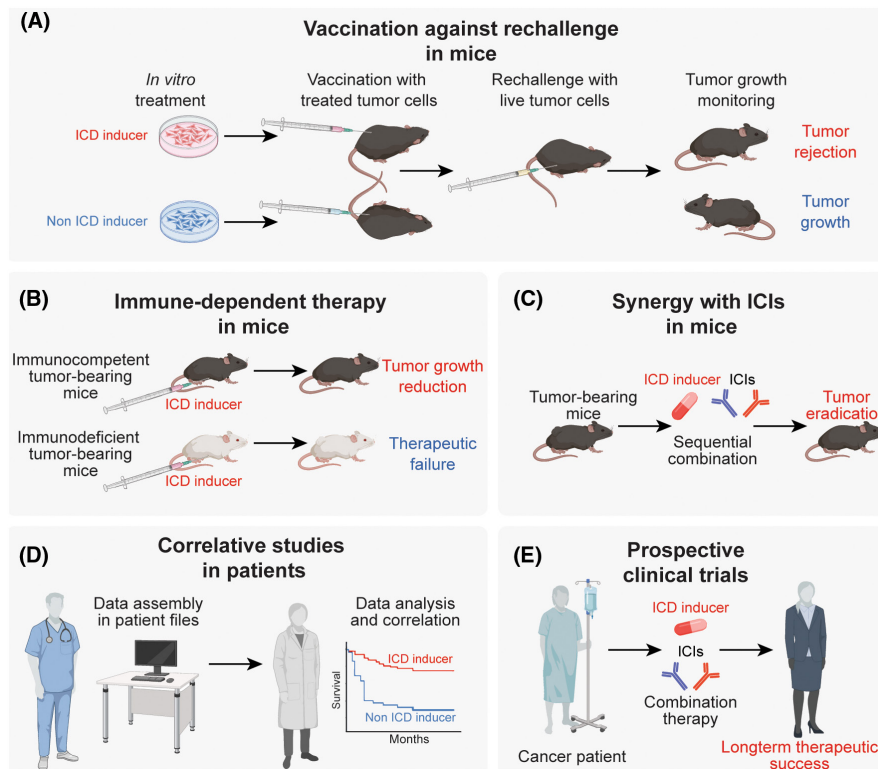


FIGURE 2 In vivo validation and clinical translation of immunogenic cell death (ICD) inducers. (A) The gold standard experiment for ICD inducer candidate drugs evaluates the efficacy of mouse cancer cells that are treated with the candidate drug in vitro and are then injected subcutaneously into syngeneic immunocompetent hosts. The mice are subsequently rechallenged with live cancer cells, usually within 7–14 days, and the absence of tumor growth is then interpreted as the sign of an immune response that leads to tumor rejection. (B) Candidate drugs can be further evaluated for their capacity to control the growth of established cancers more efficiently in immunocompetent than in immunodeficient mice (such as constitutively athymic *nu/nu* mice or mice depleted of CD4⁺ and CD8⁺ T cells). (C) ICD inducers can be favorably combined with immune checkpoint inhibitors (ICIs) including antibodies neutralizing CTLA-4, PD-1, or PD-L1. (D) Retrospective clinical studies as well as the correlative assessment of immune markers can further validate the onset of ICD. (E) Several ICD inducers that have been identified by means of the ICD discovery platform underwent prospective clinical evaluation in interventional trials and obtained approval by regulatory agencies.

T-cell hybridoma cells (which is an immortalized OT1 cell expressing a transgenic TCR specific for the ovalbumin [OVA]-derived octapeptide SIINFEKL bound to MHC Class II H-2K^b molecules) after their confrontation with DCs pulsed with the OVA protein. Although the screen had been designed in a way that the drugs would first act on DCs pulsed with OVA and then withdrawn from the system by washing, it turned out that one drug, astemizole, did not act on DCs but rather on T cells. This serendipitous finding was possible due to a carryover of astemizole to the T cells. Indeed, exhaustive, repeated washing abolished the effect of astemizole on the system.⁸⁶

In a series of exploratory experiments, we could show that astemizole did not only elicit IL2 production by B3Z cells in the absence of any cognate antigen (OVA protein or SIINFEKL peptide pulsed on DCs) or costimuli provided by DCs, but also stimulated the expression of the activation marker CD69 and the secretion of interferon- γ (IFN γ) by primary T cells isolated from the mouse spleen. Injection of astemizole into the murine food pad caused CD4⁺ and CD8⁺ T cells in the popliteal lymph node to produce IFN γ to express the T-box transcription factor TBX21 (best known as T-bet, which is necessary for the generation of TH1 and TC1 cells, that is, T cells endowed with

the capacity to produce IFN γ)⁸⁷ and to acquire activation markers (CD69 and ICOS).⁸⁶ When added to human Jurkat T cells, astemizole caused the rapid (within minutes) activating phosphorylation of three TCR-proximal signaling kinases, (i) linker for activation of T cells (LAT), (ii) lymphocyte-specific protein tyrosine kinase (LCK), and (iii) Zeta-chain-associated protein kinase 70 (ZAP70). This result was confirmed using a Jurkat clone expressing a biosensor of LCK activation. Astemizole also induced rapid Ca²⁺ fluxes in Jurkat cells. Pharmacological inhibition of LCK or chelation of intracellular Ca²⁺ prevented the astemizole-elicited upregulation of CD69, as well as the secretion of IFN γ and IL2 by B3Z cells, indicating that LCK activation and Ca²⁺ spikes are indeed required for the T-cell stimulatory effects of astemizole.⁸⁶

Astemizole has several pharmacological targets. It acts as an antagonist of histamine receptor H1 (HRH1),⁸⁸ as well as of KCNH1 and KCNH2 potassium channels.⁸⁹ Molar excess of histamine and HRH1 agonists inhibited the astemizole-elicited LCK activation in Jurkat cells, while a series of alternative HRH1 antagonist stimulated LCK activation. Histamine H1 (but not H2, H2, or H4) receptor agonists also blocked IL2 production by B3Z cells stimulated with

astemizole. Most importantly, splenic CD4⁺ and CD8⁺ T lymphocytes from HRH1 knockout mice (genotype: *Hrh1*^{-/-}) exhibited an increased spontaneous production of IFN γ as compared to WT controls, and this effect could not be further enhanced by astemizole. These results convincingly demonstrate that the primary pharmacological target of astemizole explaining its T-cell stimulatory effects is HRH1. Of note, spleens from *Hrh1*^{-/-} mice also contained reduced levels of FOXP3⁺CD4⁺ regulatory T cells as compared to wild-type controls. Hence, the available evidence indicates that the proximal target of astemizole (upstream of LCK) is HRH1 (Figure 3).

In vivo experiments involving immunocompetent sarcoma-bearing mice revealed that astemizole had no anticancer effect on its own, yet synergized with oxaliplatin in its tumor growth-decelerating and animal survival-extending effects. This effect was lost in immunodeficient mice lacking T cells. In immunocompetent mice, the combination of oxaliplatin and astemizole was particularly efficient in improving the ratio of sarcoma infiltrating CD8⁺ cytotoxic T cells over FOXP3⁺CD4⁺ regulatory T cells. Further experiments involving mice with triple-negative mammary carcinoma or orthotopic non-small cell lung cancer confirmed the capacity of astemizole to enhance the anticancer effects of oxaliplatin.⁸⁶ These findings echo a prior report on mouse models of transplantable breast carcinomas, colorectal cancers, and melanomas that exhibit particularly good responses to the dual inhibition of the PD-1/PD-L1 checkpoint (with a PD-1 antibody or *Pd-1* knockout) and HRH1 (with fexofenadine or *Hrh1* knockout).⁹⁰

There are several lines of correlative evidence suggesting that the immunostimulatory effects of astemizole on T cells are clinically relevant. First, high expression of HRH1 mRNA in tumors strongly anticorrelated with the abundance of CD4⁺ TH1 cells (which have a positive prognostic impact) but correlated with T-cell exhaustion

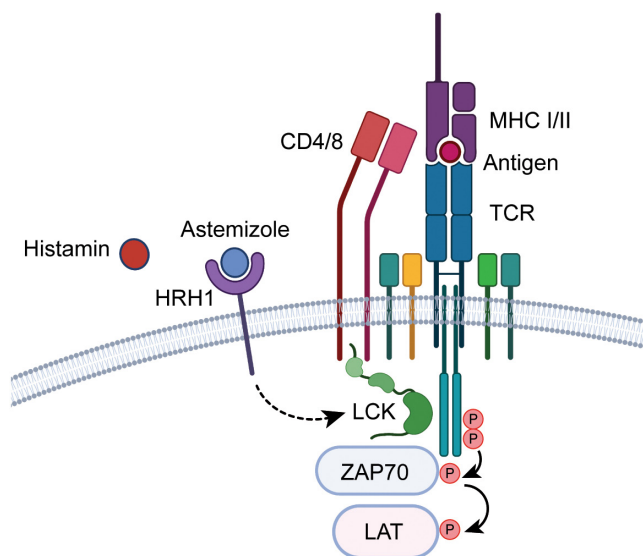


FIGURE 3 Astemizole-mediated T-cell activation. Astemizole acts as an antagonist of histamine H1 receptors (HRH1), elicits LCK activation and triggers the downstream activating phosphorylation of the T-cell receptor kinases ZAP70 and LAT, altogether increasing T-cell activation.

markers (which have a negative prognostic impact) across multiple cancer types.⁸⁶ Second, retrospective epidemiological analyses indicate that use of specific antihistamines (including astemizole) is associated to overall survival of patients with localized ovarian cancer⁹¹ and any type of metastatic cancers.⁹² Third, the use of antihistamines has been linked to favorable outcome of immunotherapy in analyses focusing on melanoma⁹³ and non-small cell lung cancer patients,⁹⁰ as well as in a pan-cancer analysis.⁹⁴ Fourth, in a basket trial involving patients with breast, colon, or lung cancer, clinical responses to PD-1 blockade negatively correlated with histamine levels in the blood.⁹⁰ Unfortunately, however, there are no published clinical trials evaluating combination effects of HRH1 antagonists with cancer chemotherapy or immunotherapy. Moreover, at the mechanistic level, it is not known whether HRH1 antagonists may overcome T-cell exhaustion.

Taken together, the aforementioned data suggest the possibility to identify T-cell stimulators in target-agnostic screens and that at least one class of such stimulators, HRH1 antagonists, have a high probability to become clinically useful. Future screening efforts should be designed accordingly.

4 | SCREENING FOR ICD ENHANCERS

As explained in the Introduction, “ICD enhancers” are pharmacological agents that act on DCs to improve their response to cancer cells (or cells infected by pathogens) that undergo immunogenic cell stress and death. We initiated the search for ICD enhancers, driven by the discovery that a frequent genetic defect in a pattern recognition receptor, formyl peptide receptor-1 (FPR1), that is due to a highly prevalent loss-of-function single-nucleotide polymorphism (rs867228, allelic frequency ~20%), causes an immune defect that selectively affects DCs, due to a failure in their chemotactic approximation to dying cells.³³ Patients bearing rs867228 develop carcinomas earlier than individuals lacking rs867228. The acceleration of cancer diagnosis amounts to ~9 months for heterozygous individuals (~30% of the population) and to ~2 years for individuals that are homozygous in rs867228 (~4% of the population).³⁸ For some cancers, these effects are particularly important. Thus, women bearing rs867228 in homo- or heterozygosity (~34% of the population) develop luminal B breast cancers 6 years earlier than women lacking rs867228 (~66% of the population).^{38,95} Accordingly, mice lacking *Fpr1* (genotype: *Fpr1*^{-/-}) developed luminal B-like breast cancer, which is under strong immunosurveillance, more rapidly than immunocompetent WT mice after exposure to a synthetic progesterone combined with DNA damage.^{38,96} Moreover, cancers established in *Fpr1*^{-/-} mice failed to respond to chemotherapy with ICD inducers (such as cyclophosphamide, doxorubicin, mitoxantrone, and oxaliplatin) that were efficient in WT mice^{33,38} echoing the observation that women with breast cancer bearing rs867228 showed a reduced progression-free and overall survival in response to adjuvant chemotherapy.³³

Driven by the consideration that the FPR1 defects causes a clinically impactful cancer-relevant immune defect, we decided

to develop an *in vitro* model to study the effect of FPR1 on DCs (Figure 4A). For this, we took advantage of conditionally immortalized immature DC line from C57BL/6 mice, in which the SV40 large T-cell antigen (SV40LgT) is expressed under the control of a Tet-On (doxycycline inducible) promoter and the reverse tetracycline transactivator is fused to the ligand-binding domain of a mutated glucocorticoid receptor. Due to the simultaneous blockade of tumor protein 53 and retinoblastoma by SV40LgT, these cells are in an inducible/immortalized state (iniDCs) in the presence of doxycycline and the glucocorticoid receptor agonist dexamethasone, yet can be de-induced/de-immortalized by the removal of both factors (de-iniDCs).²² Using the CRISP/Cas9 system, which was stably introduced into iniDCs, we knocked out all known pattern recognition receptors, then differentiated iniDCs to de-iniDCs and measured their capacity to present antigen to T cells. For this, de-iniDCs were pulsed with OVA protein, washed and cocultured with B3Z hybridoma cells, which express a transgenic T-cell receptor (TCR) recognizing the OVA-derived peptide SIINFEKL bound to MHC Class-I H-2K^b molecules. In a final step, the production of IL2 by B3Z cells was measured. In contrast to WT de-iniDCs, *Fpr1*^{-/-} de-iniDCs failed to

present OVA to B3Z cells, and this defect was the most profound one, in quantitative terms, among all pattern recognition receptors that we knocked out.³⁸ Of note, WT (but not *Fpr1*^{-/-}) de-iniDCs injected intravenously into cancer-bearing mice were able to reduce tumor growth via the activation of T-cell-dependent anticancer immune response, confirming the *in vitro* observations.³⁸

In the next step, we decided to perform a pharmacological screen to identify drugs that restore the antigen presenting function of *Fpr1*^{-/-} de-iniDCs *in vitro*. Polyinosinic-polycytidylic acid (poly(I:C)), which is synthetic analog of double-stranded RNA (dsRNA) binding to Toll-like receptor-3 (TLR3) was the sole agent in a library of pattern recognition receptor ligands capable to reverse the defect of *Fpr1*^{-/-} de-iniDCs without any effect on WT de-iniDCs. In a series of *in vivo* validation experiments, we then could show that (i) the tumor growth reducing effect of *Fpr1*^{-/-} (but not WT) de-iniDCs was enhanced by intraperitoneal injection of poly(I:C); (ii) the chemotherapy resistance of tumors implanted in *Fpr1*^{-/-} (but not WT) mice was improved by poly(I:C); and (iii) the positional defect of *Fpr1*^{-/-} DCs within the tumor bed (where *Fpr1*^{-/-} DCs are unusually distant from apoptotic cancer

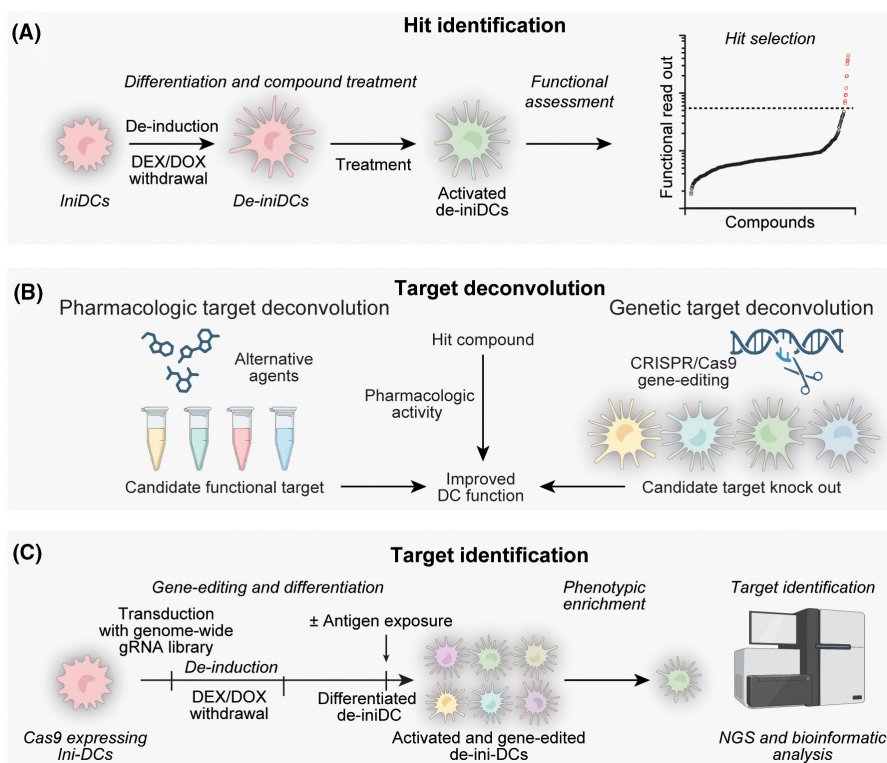


FIGURE 4 Screening for immunogenic cell death (ICD) enhancers. (A) Inducible immortalized dendritic cell (DC) precursors (iniDC) grow in the presence of doxycycline and dexamethasone but can be de-induced and differentiated into functional DCs (de-iniDCs) by the removal of both factors. De-iniDCs can be subjected to chemical compound libraries before being pulsed with antigen and functional assessment. This allows for the identification of agents that enhance the capability of DCs to integrate ICD signaling—so-called ICD enhancers. (B) In target deconvolution studies, the molecular target of ICD enhancers can be assessed by comparing the pharmacologic activity of the candidate drug to other drugs from the same chemical or functional class. Moreover, CRISPR/CAS9-mediated gene-editing can be employed to deplete the target protein from DCs and thus phenocopy the effect of the ICD enhancer (in the case of a gain-of-function phenotype) or abrogate the effect of the ICD enhancer (in the case of a loss-of-function phenotype). (C) Unbiased whole genome CRISPR/CAS9 screens can be performed on iniDC. Such screens employ barcoded gRNA and aim at identifying gain-of-function phenotypes that can be detected by flow cytometry. Next generation sequencing (NGS) can then be used to identify gain-of-function genotypes.

cells post-chemotherapy) was corrected by poly(I:C) as well.³⁸ Moreover, we could replace poly(I:C) by another fully synthetic and chemically defined TLR ligand, TL-532 (a 70 base pair dsRNA with a defined sequence),⁹⁷ replicating the restoration of the FPR1 defect *in vitro* and *in vivo*.⁹⁸

The aforementioned results suggest that de-iniDCs can be used to model DC-relevant immune defects and their immunopharmacological treatment. Based on this consideration, we have now started screening campaigns on WT de-iniDCs with the scope of identifying drugs that exert adjuvant effects, potentially by reversing DC-relevant “checkpoints.” Among the agents contained in the ICCB library of known bioactive compounds, we found ikarugamycin to be particularly active in stimulating the presentation of OVA by WT de-iniDCs to B3Z cells.⁸⁶ Ikarugamycin is an antibiotic that has been described to mediate multiple effects on mammalian cells including inhibition of clathrin-mediated endocytosis,⁹⁹ activation of the pro-autophagic transcription factor TFEB¹⁰⁰ and inhibition of the enzymatic activity of hexokinase 2.¹⁰¹ Ikarugamycin turned out to upregulate DC activation markers including CD40, CD80, and CD86 in bone marrow-derived DCs (BMDCs). Similarly, the knockdown of hexokinase caused BMDC to upregulate CD40, CD80, and CD86 and to enhance their capacity to present OVA to B3Z cells, indicating that it is most likely hexokinase inhibition that accounts for the DC-activating effects of ikarugamycin. More importantly, intratumor injection of ikarugamycin synergized with systemic oxaliplatin-based chemotherapy in stimulating tumor infiltration by mature activated (CD86⁺) DCs and in reducing cancer progression due to an anticancer immune response mediated by T lymphocytes.⁸⁶

These observations underscore the validity of the approach. Drugs that enhance the antigen presenting and T-cell activating function of de-iniDCs *in vitro* have a high likelihood to mediate immunostimulatory effects on natural DCs *in vivo*.

5 | TARGET DECONVOLUTION FOR ICD ENHANCERS

If a pharmacological agent (“agent X”) identified in a screen turns out to improve DC function in the context of ICD in validation experiments and hence acts as a bona fide ICD enhancer, it is important to understand its mode of action and hence to identify its molecular target. For target deconvolution, there are several possible strategies that we will discuss in this section.

As a first approach (Figure 4B, left), it is possible to compare the pharmacologic activity of agent X to other drugs from the same class, if there is published information on possible targets of agent X. For example, in the case of ikarugamycin, which has multiple distinct published targets (see above), it is possible to use other antibiotics, inhibitors of clathrin-dependent endocytosis, TFEB activators or hexokinase 2 inhibitors and to examine them for their ICD enhancing effects *in vitro* and *in vivo*. If only hexokinase 2 inhibitors (such as clotrimazole)¹⁰² consistently improve anticancer immune response elicited by ICD inducers, it can be concluded that it is likely

hexokinase 2 inhibition (but not any other of the ikarugamycin effects) that accounts for its immunostimulatory action. Similarly, if poly(I:C) can be replaced by other TLR3 agonists with different chemical building blocks such as poly(A:U),⁹⁸ it appears highly probable that it is indeed TLR3 inhibition that accounts for its immunotherapeutic activity.

As a second approach (Figure 4B right, C), genetic methods can be employed, again under the condition that possible targets have been identified, by suppressing the candidate target by knockout (usually using CRISP/Cas9 technology) or by knockdown (usually by RNA interference using small hairpin RNAs, shRNAs). If agent X is an activator, the elimination of the target should suppress its effects. This has been achieved for poly(I:C). Indeed, the knockout of TLR3 (but not that of other Toll-like receptors) abolished the capacity of poly(I:C) to stimulate antigen presentation by FPR1-deficient de-iniDCs *in vitro*,⁹⁸ hence proving that poly(I:C) specifically acts on TLR3 to mediate its immunostimulatory effects. If agent X is an inhibitor, the elimination of its target should mimic its effects. This has been demonstrated for ikarugamycin, which acts as a putative hexokinase 2 inhibitor. Indeed, knockdown of hexokinase 2 with two distinct, non-overlapping shRNAs improved antigen presentation of DCs *in vitro* to a similar extent as did ikarugamycin. Moreover, the combination of both procedures, namely (i) addition of ikarugamycin and (ii) knockdown of hexokinase 2 did not achieve a better immunostimulatory effect than each of the two procedures separately,⁸⁶ demonstrating that ikarugamycin solely acts on hexokinase to mediate immunostimulation.

On theoretical grounds, the strategies employed above may fail, hence leading to the conclusion that the target of the drug remains elusive. Moreover, there may be no literature-based candidate targets for new pharmacological agents identified in large compound libraries. In this case, target deconvolution may become a true challenge requiring strategies for the identification of interacting (protein) targets, orthogonal multi-omics approaches, and AI-based exploration of large datasets.^{103–105} To facilitate target deconvolution, we have started to use genome-wide CRISP/Cas9-based screens complemented by individual knockouts of candidate targets using specific guidance RNAs, to establish a catalog of several hundreds of iniDC lines that are characterized for their genotypes, as well as for their (gain-of-function, neutral, or loss-of-function) phenotypes. We plan to use this catalog of cell lines for target deconvolution by comparing their phenotypes in control conditions (without addition of agent X) with those after addition of agent X. On theoretical grounds, an immunostimulatory agent X identified to act on genetically unmodified (wild-type) de-iniDCs will fail to further improve the function of those knockout DCs that lack genes required for the pathway that is activated or de-inhibited by the agent. We believe that this approach will greatly accelerate target deconvolution, narrowing down the possible targets from the entire cellular proteome to a few proteins involved in a specific signal transduction cascade.

In conclusion, there are multiple strategies to identify the molecular targets of ICD enhancers that can be either hypothesis-driven or systematic. It is our hope that saturating genetic screens leading

to the identification of major (positive or negative) modulators of DC function will greatly facilitate target deconvolution for novel drugs with a hitherto unknown mode of action.

6 | CONCLUSIONS AND PERSPECTIVES

In the present review, we have discussed strategies for the identification of drugs that improve anticancer immunosurveillance by favorably influencing the molecular crosstalk between cancer cells, DCs, and T lymphocytes by three different effects: (i) the induction of ICD in malignant cells, hence rendering them capable of activating an innate response by DCs that culminates in the stimulation of a cognate response by T cells; (ii) the enhancement of DC function, boosting their response to stressed or dying cancer cells and their antigen-presenting or T-cell activating function; and (iii) drugs that directly act on T lymphocytes to directly cause their activation and perhaps to reduce their threshold for activation by DCs. Such drugs can be classified as (i) ICD inducers, (ii) ICD enhancers, and (iii) T-cell stimulators, respectively. We have enumerated a number of examples for each of these drug classes that have been validated in mouse experiments.

As a limitation of our approach, clinical validation for the therapeutic utility of our concept has only been obtained for ICD inducers in prospective clinical trials,^{72-76,80} while most of the evidence is based on retrospective analyses.^{13,26,61-70} Clinical evidence in favor of the use of T-cell stimulators is based on retrospective observations and is merely correlative.^{86,90-94} Finally, there is no strong clinical evidence yet in favor of the potential utility of ICD enhancers for cancer treatments. Several randomized trials using anticancer therapeutic vaccines have demonstrated potent adjuvant effects for TLR3 agonists, but none of these trials revealed actual clinical benefit.¹⁰⁶⁻¹⁰⁹ Hence, the concept of ICD enhancers will require further scrutiny with respect to its potential clinical implications.

Notwithstanding these limitations, strongly encouraged by our successful participation in screening campaigns leading to the development of clinically validated (and FDA/EMA-approved) ICD inducers, we will continue our efforts to pinpoint ICD enhancers and T-cell stimulators for anticancer immunotherapy. Indeed, on theoretical grounds, ICD inducers (which act on cancer cells) can easily elicit resistance mechanisms that come into action due to heterogeneity and (epi-) genetic plasticity of malignant cells. In contrast, ICD enhancers and T-cell stimulatory would directly act on immune cells, hence harboring higher chances to mediate broad immunotherapeutic effects against an array of distinct malignant diseases.

Thus far, our screening approaches have relied on the investigation of single cell types (cancer cells, DCs, and T cells) or combination of two cell types (e.g., cancer cells confronted with DCs, DCs cocultured with T cells, T cells probed for cytotoxic activity against cancer cells). We are currently examining the possibility to combine all three cell types in one single assay to elaborate a sort of “mini-immune system.” We have learned in the past that the spatial FPR1-regulated localization of DCs with respect to cancer cells plays a major role

in determining the outcome of anticancer immune responses.^{33,38} Moreover, it appears that the spatial organization of immune cells in so-called tertiary lymphoid organs is indispensable for the tumor immune contexture and the clinical outcome of immunotherapy.¹¹⁰ For this reason, it will be important to experimentally modify and characterize the tridimensional (3D) arrangement of cell types present in the “mini-immune system.” We are currently assembling a 3D spatial biology platform combining sophisticated 3D bioprinting with the kinetic monitoring by confocal fluorescence microscopy of artificial immune systems cultured in a 3D matrix.

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CONFLICT OF INTEREST STATEMENT

OK and GK have been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Biopharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Tollys, and VAScage. GK is on the Board of Directors of the Bristol Myers Squibb Foundation, France. GK is a scientific co-founder of EverImmune, Osasuna Therapeutics, Samsara Therapeutics, and Therafast Bio. OK is a scientific co-founder of Samsara Therapeutics. GK is in the scientific advisory boards of Hevolution, Institut Servier and Longevity Vision Funds. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis, and metabolic disorders. GK's brother, Romano Kroemer, was an employee of Sanofi and now consults for Boehringer-Ingelheim. LZ has held research contracts with GlaxoSmithKline, Incyte, Lytix, Kaleido, Innovate Pharma, Daiichi Sankyo, PiLeje, Merus, Transgene, 9M, Tusk and Roche, was on the on the Board of Directors of Transgene, is a cofounder of EverImmune, and holds patents covering the treatment of cancer and the therapeutic manipulation of the microbiota. The funders had no role in the design of the study, in the writing of the manuscript, or in the decision to publish the results. The other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

This paper does not include any new data.

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