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EDITORIAL

An unexpected link between immunogenic cell death and inhibition of gene transcription

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Colliding with the ancient concept that chemotherapeutics may delay (and sometimes stop or even reverse) the advancement of cancer because they kill malignant cells, it has become clear that successful treatments with cytotoxicants (and presumably also with some of the so-called targeted agents) only have durable effects when they succeed in stimulating an anticancer immune response. This discovery was spurred by preclinical experiments, unraveling that anthracyclines and other cytotoxicants are much more efficient in controlling the growth of tumors evolving in immunocompetent (as opposed to immunodeficient) mice and then validated in cancer patients in which chemotherapy-induced changes in the immune infiltrate predict the therapeutic response.[1,2] One major mechanism through which chemotherapy induces clinically relevant anticancer immunity resides in their capacity to induce immunogenic cell death (ICD), meaning that they kill tumor cells in a way that they become recognizable to the immune system.

Unfortunately, only a fraction of chemotherapeutic agents is capable of stimulating ICD, meaning that only some particularly efficient anticancer agents (such as anthracyclines for breast cancer or oxaliplatin for colon cancer) are able to do so.[3,4] This observation has motivated us and others to define the particularities of pharmacological ICD inducers (as compared to non-ICD inducers), leading to the discovery that ICD inducers are endowed with the ability to stimulate a series of premortem stress responses that adjuvantize cancer cells, hence alerting innate immune effectors, in particular dendritic cells (DC) and their precursors. These peculiar ICD-associated stress responses involve autophagy (which facilitates the lysosomal release of ATP from dying cancer cells, causing the emission of a chemotactic signal for DC precursors) as well as the activation of the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), a hallmark of endoplasmic reticulum (ER) stress (which facilitates the exposure of the normally ER-resident protein calreticulin to the cell surface, where calreticulin then serves as an 'eat-me' signal to facilitate the engulfment of tumor antigens by immature DC.[5] Of note, recent work reveals that eIF2α phosphorylation is also required for autophagy induction in some contexts,[6] suggesting that both hallmarks of ICD (ATP release and calreticulin exposure) may be mechanistically linked. Additional hallmarks of ICD include the induction of a type-1 interferon response (to stimulate the recruitment of cytotoxic T lymphocytes into the tumor immune infiltrate) as well as the release of annexin A1 and high mobility group A1 (HMGB1) protein from the cytoplasm and nuclei of dead cells respectively, to stimulate correct DC positioning and DC maturation in the tumor bed.[1,4]

Based on these insights, we have built medium-throughput high-content screening strategies in which human osteosarcoma U2OS cancer cells are equipped with suitable biosensors (to measure ATP release, calreticulin exposure, type-1 interferon signaling and HMGB1 release), then cultured with compound collections, and characterized for the induction of ICD characteristics, followed by validation experiments in vitro (with other methods and on other cell lines) and in vivo (to measure the effective induction of anticancer immune responses in mouse models).[7,8] Several large screens including the measurement of additional stress responses (like all arms of the ER stress response) led to the generation of a data bank, instructing us on the facts that (i) induction of other types of ER stress than eIF2α phosphorylation was not activated upon ICD induction and (ii) that anticancer drugs must have a specific set of physicochemical characteristics that define their propensity to induce ICD, allowing to use artificial intelligence to create a mathematical model, which, based on molecular descriptors, yield a theoretical 'ICD score'.[5] When computing this ICD score to a library of 50,000 agents, we found that one of the top hits was dactinomycin (DACT, best known as actinomycin D), an agent that has been used by myriads of molecular biologists to inhibit transcription but that is also employed for the chemotherapy of sarcomas. Intrigued by this observation, we used multiple tests to measure RNA synthesis (and downstream protein synthesis, downstream of RNA transcription) to conclude that most ICD inducers (including anthracyclines, oxaliplatin, lurbinectedin, crizotinib and thiostrepton) actually cause an inhibition of DNA-to-RNA transcription (and hence

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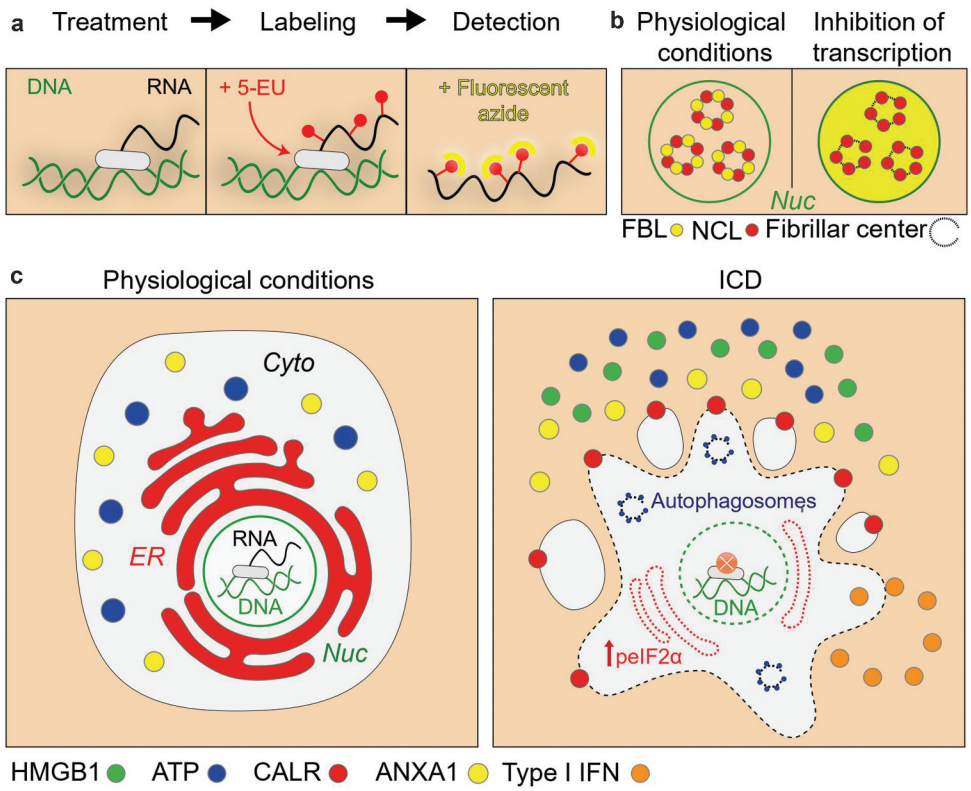

Figure 1. Principle of the measurement of transcription inhibition. **A**. Following the treatment with an experimental compound, transcription can be analyzed by quantifying the incorporation of click-it chemistry-detectable 5-ethynyl uridine (EU). Incorporated EU molecules can be microscopically visualized with fluorescently labeled azide upon fixation.**B**. Alternatively, cells can be fixed after treatment and stained with antibodies specific to nucleolin (NCL) and fibrillarin (FBL) which facilitate RNA synthesis in the fibrillar center of the nucleus. Thus, the absence of colocalization of these two proteins is indicative for impaired transcription.**C**. The emitted pool of immunogenic cell death (ICD)-related danger associated molecular patterns includes endoplasmic reticulum (ER)-resident calreticulin (CALR), whose exposure at the cell surface depends on eIF2α phosphorylation (peIF2α), ATP that is released in an autophagy-dependent fashion, the production of type I interferon (IFN) as well as the exodus of high mobility group box 1 (HMGB1) from the nucleus (*Nuc*) and annexin A1 (ANXA1) from the cytoplasm (*Cyto*) during cell death. For the purpose of drug discovery, the hallmarks of ICD, including the inhibition of DNA-to-RNA transcription, can be measured by click-it chemistry such as 5-ethynyl uridine (EU)-containing RNA or biosensor cell lines expressing LC3 (present in the membranes of autophagosomes), CALR or HMGB1 fused to a fluorescent protein. Other hallmarks such as the release of ATP can be detected with the chemical dye quinacrine, ANXA1 released in the supernatant can be measured by ELISA and the production of type I IFN can be monitored on the mRNA level.

a subsequent inhibition of RNA-to-protein translation) and that this effect may cause a peculiar ER stress response consisting in the phosphorylation of eIF2α by eIF2α kinase 3 (EIF2AK3, better known as PERK) without any other signs of the unfolded stress response such as activation of the ATF6 and the IRE1-XBP1 axes.[8–10] The aforementioned results suggest that a vast class of ICD inducers (with the exception of microtubular poisons such as vinca alkaloids and taxanes) is able to reduce RNA synthesis, which resembles a response to viral infection. This has two major implications. On one hand, it is possible to consider anticancer agents with known transcription-inhibitory properties as candidates for ICD induction.[9] On the other hand, it is relatively easy to measure inhibition of RNA synthesis in vitro, on cultured cells, for example using a chemically derivatized uridine analogue whose incorporation into nascent RNA, can be visualized by click chemistry to yield a fluorescent signal (Figure 1a). An alternative method to diagnose stalled transcription consists in detecting the separation of two proteins involved in RNA synthesis, nucleolin and fibrillarin, that normally (when RNA synthesis is active) colocalize in the nucleus, yielding an overlapping immunofluorescence staining (Figure 1b).[9]

Hence, these types of tests might be easily added to the current compendium of assays to measure immunogenic stress events induced by anticancer agents (Figure 1c), adding yet another criterion to discriminate agents with a high potential of ICD induction from agents with a lower probability to kill cancer cells in an immunogenic fashion. Thus, this information could be fed into existing and yet-to-be-developed databanks to improve the algorithm calculating the 'ICD score', further refining the approach leading to the identification of ICD inducers.

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Disclosure of Potential Conflicts of Interest

GK and OK are cofounders of Samsara Therapeutics.

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