Chemotherapy and radiotherapy: Cryptic anticancer vaccines

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An attractive, yet hitherto unproven concept predicts that the promotion of tumor regression should elicit the host’s immune response against residual tumor cells to achieve an optimal therapeutic effect. In a way, chemotherapy or radiotherapy must trigger “danger signals” emitted from immunogenic cell death and hence elicit “danger associated molecular patterns” to stimulate powerful anticancer immune responses. Here, based on the recent experimental and clinical evidence, we will discuss the molecular identity of the multiple checkpoints that dictate the success of “immunogenic chemotherapy” at the levels of the drug of the tumor cell and of the host immune system.

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1. Introduction

In the 1990s, the genetic engineering of recombinant viral vectors facilitated the emergence of a novel concept of anticancer vaccines that prevailed until recently. Indeed, irradiated genetically modified autologous or allogeneic tumor cells were broadly utilized in preclinical studies and clinical trials to elicit tumor-specific humoral and cellular immune responses that were occasionally associated with tumor regression [1]. To enhance the potency of antitumor immunity, several groups devised strategies to augment the uptake and cross-presentation of dying tumor cells by dendritic cells (DCs). Among these, one of the most successful was that developed by Dranoff and colleagues, who vaccinated with irradiated tumor cells (cell lines or autologous dissociated tumor pieces) that were engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) by means of recombinant retroviruses or adenoviruses. Such dying cells were able to mobilize DC, plasma cells, invariant NKT cells and tumor reactive CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, both in mice and cancer patients, alone or in conjunction with anti-CTL\textsubscript{A}4 Ab [2]. Importantly, GM-CSF expressing dying tumor cells could promote tumor destruction, necrosis and fibrosis correlating with humoral immune responses and favorable clinical outcome [3,4]. The comprehensive analyses of the specificities recognized by the post-vaccine IgG antibodies revealed key autoantigens involved in cell cycle regulation, cellular stress and oncogenesis [5–9].

Unfortunately, the state-of-the-art GMP conditions required to freeze–thaw irradiated tumor cells before injection into patients in Phase III trials, jeopardizing the immunogenic potential of the vaccine while suggesting that the specific characteristics of dying cells dictate the clinical outcome (Pardoll and Dranoff, personal communications). Indeed, Albert et al. reported that apoptotic cell death could be immunogenic by facilitating antigen cross-presentation by DC, a phenomenon that could be relevant to the pathogenesis of autoimmune paraneoplastic syndromes [10,11]. These findings inaugurated the debate on how cellular death, whether necrotic, apoptotic, autophagic, senescent or associated with mitotic castastrophe, may generate tolerance, ignorance or immunity [12,13] and how this knowledge might be exploited to generate optimal cancer vaccines.

2. Cell death inducers do not always mediate immunosuppression and can synergize with immunomodulators

The effects of anticancer drugs on the immune system have been detailed in previous reviews [14,15]. As a reminder, radia-
tion therapy (either as a single or fractionated dose) can induce tumor-specific Th1 and Tc1 cells in the draining lymph nodes of the irradiated tumor and even favors the trafficking of effector cells into tumors in an IFN-γ-dependent manner [16,17]. Moreover, potent synergistic effects against established tumors between passively transferred CTLs [18], intratumoral DC [19] or TLR9 ligands [20] and ionizing radiation have been reported. A recent translational study performed on colon and prostate cancer patients undergoing radiation therapy (plus or minus chemotherapy) revealed that survivin-specific CD8+ T cell responses, which were already detectable in 50% cases prior to therapy, increased significantly post-therapy in the vast majority of patients. Moreover, in those responding to the therapeutic regimen, higher levels of nuclear (rather than cytoplasmic) survivin were detected in tumor beds [21].

Interestingly, not only cancer cells but also stromal cells can be directly targeted by chemotherapy-induced CTLs [22]. Hans Schreiber and coworkers elegantly showed that in cases of low antigen expression by tumor cells, antigen transfer to stromal cells is mandatory for complete cure. Treating advanced tumors with local chemotherapy or radiotherapy caused the transfer of tumor-specific MHC/peptide complexes to stromal cells, allowing adoptively transferred CTLs to efficiently attack the tumor [22].

The host immune system also contributes to the effects of so-called “targeted” therapies that have been recently added to the oncological armamentarium. In combination with intratumor inoculation of adenoviruses engineered to express IL-12 and 4-1BB, daily administration of the tyrosine kinase inhibitor sunitinib significantly improved long-term survival in mice bearing large tumor burdens while each therapeutic approach alone failed to mediate antitumor efficacy [23].

Finally, classic immunization and chemotherapeutical strategies can synergize. Using DNA-based-vaccination targeting an oncogenic protein involved in tumor maintenance, Chiarle et al. demonstrated that plasmids encoding the cytoplasmic domain of anaplastic lymphoma kinase (ALK) can immunize mice against anaplastic large cell lymphoma (ALCL) in a CD8+ and IFN-γ-dependent manner and cure animals bearing advanced ALCL when combined with doxorubicin [24].

Correale et al. pioneered the field of chemoimmunotherapy in Phase II trials launched in metastatic colon cancer by combining immunogenic chemotherapy (gemcitabine + oxaliplatin) with GM-CSF and IL-2 [25,26] and showed that tumor antigen-specific immune responses and autoimmune side effects can accompany encouraging clinical outcome [27].

These examples illustrate that radiotherapy or chemotherapy can elicit anticancer immune responses or cooperate with tumor vaccines, in line with the notion that conventional anti-neoplastic therapies may be compatible with therapeutically relevant antitumor immune responses.

3. Checkpoints for tumor immunogenicity at the drug level

Some apoptosis-inducing agents or cytotoxic anticancer drugs may directly or indirectly boost the immune system, in three different ways [14,15]. Firstly, some therapeutic regimen can elicit specific cellular responses that render tumor cell death immunogenic. Secondly, some drugs may have off-target effects that stimulate the immune system, for instance by transient lymphodepletion, by the subversion of immunosuppressive mechanisms, or by direct or indirect stimulatory effects on immune effectors. Thirdly, some drugs can sensitize tumor cells to lysis by CTL or NK cells. Here, we will focus our discussion on drugs that elicit T cell responses against tumor cells.

3.1. Systemic screenings

Tanaka et al. examined the biological effects of 54 chemotherapeutic agents on DC functions (maturation and APC function, survival and growth) using a DC biosensor system (DC line XS106 expressing the yellow fluorescent protein under the control of the IL-1β promoter) [28]. This unbiased functional screen unveiled a striking diversity among anticancer drugs. Most topoisomerase inhibitors and antimicrotubule agents promoted DC maturation. In contrast, alkylating agents, anitmetabolites, platinum-based compounds and hormonal agents failed to do so. The Vinca alkaloid vinblastine was the most efficacious in inducing CD40, CD80, CD86 and MHC class II expression on mouse and human DC and in stimulating the secretion of IL-1β, IL-6 and IL-12p40. At low dosages (0.1–1 μM), vinblastine markedly improved the uptake of FITC-dextran, antigen cross-presentation and allogeneic or tumor antigen-specific T cell responses in vivo, specifically in tumor bearing hosts [28,29]. Vinblastine mediated more pronounced antitumor effects against B16 melanoma in immunocompetent mice than in immunocompromised littermates, while the antitumor effects of cisplatin were indistinguishable in both groups [29]. These results suggest that partial or temporal disruption of the intracellular microtubule network may be sensed by DC as an immunostimulatory signal.

Our groups also performed a systematic screening of anticancer compounds for their ability to induce immunogenic cancer cell death. For this study, CT26 colon cancer cells were treated with a panel of chemotherapeutic agents that all induced 70 ± 10% of apoptosis (assessed by staining with and Annexin V). Then, the dying or dead cells were inoculated subcutaneously, in the absence of any adjuvant, into one flank of immunocompetent syngeneic BALB/c mice, which were rechallenged one week later with injection of live CT26 cells in the opposite flank. The absence of tumor growth was then scored as an indication of a productive anticancer immune response. Some 20 different apoptosis-inducing agents that operate through distinct modes of action failed to induce immunogenic cancer cell death. This applied to drugs that kill cancer cells through mitochondria, lysosomal stress, as well as tyrosine kinase inhibitors, proteasome inhibitors or DNA-damaging agents (alkylating agents or topoisomerase inhibition). In sharp contrast, anthracyclines (daunorubicin, idarubicin, mitoxanthrone) were the most potent inducers of immunogenic cell death, not only in CT26 tumors, but also in EL4 thymomas and MCA205 sarcomas [30,31]. Anthracyclines, whose chemical structure is based on sarinamide and tetra-hydro-naphthacene-dione, inhibit DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand, thus preventing the replication of rapidly growing cancer cells. They also create iron-mediated free oxygen radicals that damage the DNA and cell membranes. We found that doxorubicin could elicit immunogenic signals on enucleated tumor cells [31], suggesting that the immunologically relevant changes induced by anthracyclines are cytoplasmic. Indeed, we found that anthracyclines can elicit the rapid (within hours) phosphorylation of the eukaryotic (translation) initiation factor 2α (eIF2α), through the activation of the eIF2α kinase PERK [32] and the dissociation of the eIF2α phosphatase complex composed by PPI and GADD34 [33]. In general, it appears that chemotherapeutic agents that stimulate eIF2α phosphorylation, which is a sign of endoplasmic reticulum (ER) stress, are particularly efficient in eliciting immunogenic cell death. Based on these considerations, as well as on the molecular identification of immunogenic signals emanating from dying cells, we are currently devising high-throughput screens for the identification of drugs that can induce immunogenic (as opposed to non-immunogenic) cell death (Figs. 1 and 2).
Fig. 1. Biosensor cell lines for the measurement of immunogenic signals. (A) Schematic representation of the redistribution of GFP-CRT fusion protein from a diffuse perinuclear localization within the endoplasmic reticulum to a peripheral cytoplasmic puncta. Note that this redistribution of GFP-CRT can occur before cells manifest morphological signs of apoptosis, in which case it predicts immunogenic cell death. (B) Redistribution of GFP-HMGB1 from the nucleus to the cytoplasm and to the extracellular milieu. The release of GFP-HMGB1 occurs when secondary necrosis (necrosis after apoptosis) becomes manifest and the plasma membrane is permeabilized. (C) Release of ATP allowing a luciferase enzyme tethered via a GPI anchor to the plasma membrane to emit photons upon hydrolysis of luciferin.

3.2. Metronomic cyclophosphamide

In mice, low (so-called “metronomic”) doses of the alkylating agent cyclophosphamide potentiate delayed-type hypersensitivity (DTH) responses by acting on a cyclophosphamide-sensitive suppressor T cell subset [34]. Metronomic cyclophosphamide decreases the number and inhibitory function of CD4+CD25+ regulatory T (Treg) cells [35,36]. The cyclophosphamide-stimulated IFN-γ production might account for the augmented antibody responses and the persistence of memory T cells [37,38]. All these effects may contribute to the eradication of immunogenic tumors in synergy with specific immunotherapies [39–41]. Early clinical trials performed on a limited number of patients indicate that the combination of metronomic dosing of cyclophosphamide with vaccines do augment DTH responses [42], decrease the frequency of circulating CD4+CD25+ suppressor T cells [43] and prolong the survival of metastatic cancer patients [44]. This contrasts with recent trials using intravenous 300 mg/m²/day of cyclophosphamide that failed to reduce the number or functional activity of tumor-induced regulatory T cells [45]. Nonetheless, one daily administration of oral cyclophosphamide for one month to end-stage cancer patients significantly reduced peripheral Treg numbers and inhibited the suppressive action of Treg cells on both T and NK cells [46]. Interestingly, another alkylating agent, dacarbazine, was shown to enhance memory CD8+ T cell responses to peptide vaccines in melanoma patients, suggesting that a diverse array of alkylating agents may mediate immunostimulatory functions [47].

3.3. Gemcitabine

Gemcitabine, a synthetic pyrimidine nucleoside analogue, induced sizeable T cell responses against established hemagglutinin (HA)-transfected AB1 tumors in BALB/c mice [48] and mediates synergistic antitumor effects with a CD40 ligand [49]. Likewise, gemcitabine acts at two levels to mediate tumor immuno-
Tyrosine kinase inhibitors of ionizing irradiation accounts for the abscopal effect. Finally, promote T cell trafficking towards irradiated tumor sites, which is mediated by the immune system. Low doses of ionizing irradiation modulate the repertoire of tumor-derived peptides [18] upregulate the expression of MHC class I molecules, tumor-associated antigens [52] and CD95/Fas on tumor cells, thereby boosting CTL activity [53], and finally promote T cell trafficking towards irradiated tumor sites [16]. At present, it is not known which among these multiple effects of ionizing irradiation accounts for the abscopal effect.

3.4. Ionizing irradiation

Local irradiation of a single tumor site can induce the reduction of non-irradiated metastases located at a distant site, a phenomenon known as "the abscopal effect", which is mediated by the immune system. Low doses of ionizing irradiation modulate the repertoire of tumor-derived peptides [18] upregulate the expression of MHC class I molecules, tumor-associated antigens [52] and CD95/Fas on tumor cells, thereby boosting CTL activity [53], and finally promote T cell trafficking towards irradiated tumor sites [16]. At present, it is not known which among these multiple effects of ionizing irradiation accounts for the abscopal effect.

3.5. Tyrosine kinase inhibitors

Our own studies indicate that the paradigmatic c-kit tyrosine kinase inhibitor imatinib mesylate (IM) boosts IFN-γ secretion by NK cells, both in mice and in GIST patients [54,55]. The effects of IM on cognate immune responses have been reviewed elsewhere [56] stressing that IM does not prevent and even boosts peptide specific vaccines in chronic myeloid leukemia. Other groups investigated the immunological effects of the two novel multi-kinase inhibitors, sorafenib and sunitinib, which both have been successfully introduced in the treatment of patients with renal cell carcinoma (RCC). While sorafenib inhibited the function of DC and markedly reduced antigen-specific T cell responses in vivo [57], sunitinib reduced the numbers of circulating Treg [57,58] and myeloid derived suppressor cells, restored the production of IFN-γ by T cells, and downregulated the suppressive microenvironment of tumor beds [23,59]. However, the clinical responses of RCC patients to sunitinib did not correlate with any of the immunological parameters investigated thus far [58]. Therefore, it remains to be determined whether sunitinib might exert part of its therapeutic effect via the immune system.

4. Checkpoints for the immunogenicity of cell death at the tumor level

Chemotherapy might fail because tumor cells do not die in response to the therapeutic insult or because cell death occurs in a non-immunogenic manner, meaning that the immune system is not mobilized by the tumor cell distress. The molecular dialogue between cellular damage and innate effectors, which culminates in tumor antigen-specific cognate T cell responses, is being progressively unraveled.

4.1. Eat-me signal: calreticulin

Obeid et al. demonstrated that anthracyclines, oxaliplatin and ionizing irradiation have the potential to trigger immunogenic cell death by regulating the translocation of an ER resident protein complex (composed of calreticulin (CRT) and the disulfide isomerase ERp57) to the plasma membrane of tumor cells [31,59]. CRT/ERp57 is considered as an eat-me signal that is required for DC to engulf dying tumor cells, thereby eventually inducing T cell-dependent chemotherapeutic effects against tumors [31,60,61]. CRT exposure occurs well before the cells exhibit phosphatidylserine residues, and is abolished by blockade of ER calcium efflux [62] or caspase inhibition [31]. CRT exposure results from an ER stress response that results in the phosphorylation of eIF2α (see above). Downstream of the ER stress response, a subapoptotic event causes partial caspase-8 activation, Bap31 cleavage and conformational changes in Bax and Bak that are usually associated with apoptosis. Next, CRT/ERp57 complexes appear at the cell surface as a result of their SNARE-dependent exocytosis following an anterograde ER-Golgi trafficking of CRT/ERp57-containing vesicles [32]. Therefore, an entirely new class of proteins that have no significant impact on cell death, yet determine whether immunogenic CRT exposure occurs, could influence the clinical outcome of chemotherapy. When ERp57-deficient tumors (which cannot expose CRT at the cell surface) are implanted in mice, they are resistant against anthracycline-based chemotherapy unless exogenous CRT is injected [59]. This contrasts with the cell-autonomous response of ERp57-deficient cancer cells, which respond normally to anthracyclines in vitro. Hence, the failure to emit immunogenic signals can result in ineffective chemotherapy responses. We suspect that the expression levels and phosphorylation status of key players of the CRT exposure pathway (such as PERK, eIF2α, caspase-8, Bap31 and others) and apoptotic regulation (such as Bcl-2 family proteins and IAP proteins) might ultimately lead to an algorithm that predicts anticancer immune responses elicited by chemotherapy or radiotherapy.

4.2. Don’t eat me signal: CD47

CD47 is an Ig-like protein known to functionally interact with integrins and thrombospondin-1. It is also interacting with its receptor SIRP-α on macrophages to negatively regulate phagocytosis [63]. CD47 is constitutively upregulated on mouse and human myeloid leukemias, and overexpression of CD47 favors disease dissemination by evading macrophage-mediated phagocytosis [64]. It has been shown that the pro-phagocytic effects of plasma membrane-exposed CRT are counteracted by the expression of CD47 on the same cell that exposes CRT [65]. Interestingly, cross-linking of CD47 on a chronic lymphocytic leukemia could induce caspase-independent cell death [65,66]. Moreover, an antibody that blocks anti-CD47 was able to elicit macrophage-mediated phagocytosis of non-apoptotic CD47th human acute lymphoblastic leukemia cells [67]. On theoretical grounds, preventing the interaction between CD47 and SIRP-α could enforce the phagocytosis of tumor cells by professional APC such as DC. Whether this is the case requires urgent confirmation. Moreover, it remains an open conundrum whether inhibition of CD47 can stimulate an anticancer immune response.

4.3. Anti-inflammatory factor: milk fat globule epidermal growth factor VIII

Through studies performed in GM-CSF-deficient mice, Dranoff and Tahara identified milk fat globule epidermal growth factor VIII (MFG-E8, also called lactadherin) as a critical determinant of the pro-versus anti-inflammatory properties of GM-CSF [68]. GM-CSF induces the secretion of MFG-E8 from resting (that is nonTLR-induced) phagocytes. MFG-E8 binds to phosphatidylserine-expressing dying cells, and signals through αvβ3 and αvβ5 integrins to promote the uptake of apoptotic cells and the secretion of TGF-β and CCL22 by myeloid cells, all contributing to the maintenance of Foxp3+ Treg. In addition, MFG-E8 expression and secretion is induced in tumor cells exposed to cytokotoxic compounds and represents a potent anti-apoptotic event. Blocking antibodies to MFG-E8 could subserve four independent functions that might explain their marked synergistic anticancer effects when combined with chemotherapy, radiotherapy and molecular targeted compounds [69]. First, anti-MFG-E8 antibodies increase the susceptibility of tumor cells to drug-induced apoptosis [69]. Second, they facilitate the FcR-mediated uptake of dying cells by DC, thereby...
and then stimulate DC and promote antigen-specific CD8+ T cell responses. Dangers signals that are released by dying mammalian cells furthered the field demonstrating that monosodium urate crystals of apoptotic cells by certain macrophage subpopulations [90]. The receptor tyrosine kinase Mer is involved in the phagocytosis of apoptotic cells (clearance deficiencies), secondary necrosis can occur and HMGB1-nucleosome complexes are released from dead cells. Such HMGB1-nucleosome complexes cause the maturation of macrophages and DC (secretion of IL-1β, IL-6, TNFα, IL-10), thus breaking tolerance to dsDNA in a TR2-dependent manner [76]. Adding some more complexity, Kazama et al. succeeded in switching tolerogenic into immunogenic cell death by inducing post-transcriptional modifications in HMGB1 using a ROS scavenger. Indeed, in splenocytes undergoing apoptosis, activated caspase-3 and caspase-7 cleave the p75 kDa subunit of the respiratory complex leading to production of ROS which oxidize Cys106 in HMGB1, disabling its potential to activate DC [76]. Hence, the avoidance of HMGB1 oxidation may have immunostimulatory effects. The clinical relevance of HMGB1 and its post-transcriptional changes remain to be established in patients undergoing anticancer chemotherapy.

4.4. DAMP: HMGB1

High mobility group box 1 protein (HMGB1) is an abundant nuclear protein that is tightly associated with chromatin and acts as a transcription factor, when present in the nucleus, as well as a pro-inflammatory cytokine, when it is released from cells [71]. Damaged and necrotic cells were primarily shown to release HMGB1 into the extracellular milieu, where it triggers an inflammatory response to necrosis. Necrotic fibroblasts derived from hmg1−/− mice failed to induce the maturation of DC, in conditions in which necrotic cells from wild type mice were able to trigger DC maturation. Inhibitors of HMGB1 (such as neutralizing Ab or the HMGB1 inhibitory fragment box A) hampered the reactivity of APC to necrotic cells. Moreover, apoptotic lymphoma cells were poorly immunogenic unless they were combined with supernatants from necrotic fibroblasts. The “adjuvanticity” of the necrotic cell-derived supernatants was partially ablated by HMGB1 blockade [72]. RAGE was reported to be the receptor for HMGB1 in these experiments. Recently, several groups reported that apoptotic cells also extrude HMGB1 into the extracellular milieu [73–75], thereby mediating an immunogenic cell death pathway. Indeed, binding of HMGB1 to TLR4 on DC facilitates the processing and presentation of tumor antigens by DC-derived MHc class I molecules. This effect could be attributed to the TLR4-dependent inhibition of the lysosome-dependent degradation of the phagocytic cargo, resulting in improved tumor antigen cross-presentation [75].

In the context of impaired phagocytosis of apoptotic cells (clearance deficiencies), secondary necrosis can occur and HMGB1-nucleosome complexes are released from dead cells. Such HMGB1-nucleosome complexes cause the maturation of macrophages and DC (secretion of IL-1β, IL-6, TNFα, IL-10), thus breaking tolerance to dsDNA in a TR2-dependent manner [76]. Adding some more complexity, Kazama et al. succeeded in switching tolerogenic into immunogenic cell death by inducing post-transcriptional modifications in HMGB1 using a ROS scavenger. Indeed, in splenocytes undergoing apoptosis, activated caspase-3 and caspase-7 cleave the p75 kDa subunit of the respiratory complex leading to production of ROS which oxidize Cys106 in HMGB1, disabling its potential to activate DC [76]. Hence, the avoidance of HMGB1 oxidation may have immunostimulatory effects. The clinical relevance of HMGB1 and its post-transcriptional changes remain to be established in patients undergoing anticancer chemotherapy.

4.5. DAMP: uric acid

In certain conditions of cell stress, an endogenous adjuvant activity is delivered to the environment of the damaged tissues, influencing the inflammatory and immune outcome. Shi et al. pioneered the field demonstrating that monosodium urate crystals are danger signals that are released by dying mammalian cells and then stimulate DC and promote antigen-specific CD8+ T cell responses [77]. During chemotherapy by bleomycin, a selective inhibitor of DNA synthesis used to treat a variety of human malignancies, oxidative damage and cell death of alveolar macrophages and epithelial cells create acute lung injury culminating in interstitial pulmonary fibrosis. Gasse et al. showed that uric acid is the danger signal activating the Nlrp3 inflammasome leading to IL-1β release and IL-1R1/Myd88-dependent lung fibrosis [78,79]. In spite of these insights, it remains elusive whether uric acid has a positive or negative effect on chemotherapy-induced anticancer immune responses.

4.6. DAMP: HSP70–HSP90

A common adaptive response to cell stress, including that induced by chemotherapy, is the transcriptional activation of a series of molecular chaperones that belong to the class of inducible heat-shock proteins (HSPs). Such HSPs protect against cell death by refolding damaged proteins, by directing damaged proteins to proteasome-mediated degradation and finally by inhibiting apoptosis [80]. HSPs can also stimulate the immune system by acting on the scavenger receptor CD91 on the surface of DCs, thereby transmitting a maturation signal [81] or by chaperoning tumor-specific antigens to MHC class I and II pathways for efficient T cell activation as detailed elsewhere [15,82]. In human myeloma cells treated with the proteasome inhibitor bortezomib [83], HSP90 appears on the surface of tumor cells and serves as a contact-dependent activation signal for autologous DCs.

4.7. Pro-inflammatory and find-me signal: ATP

The systematic screening of various anticancer drugs inducing cell death with distinct mechanisms on a variety of cancer cell lines revealed that cell death is accompanied by a reduction of intracellular ATP concentrations and an accumulation of extracellular ATP [84]. Chemotherapy affects ATP levels at the pre-apoptotic level, before and during the entry of cells into the step-wise process leading to apoptosis and secondary necrosis [85]. It remains to be determined whether ATP is passively or actively exocytosed (via vesicular trafficking) from cancer cells undergoing the chemotherapeutic hit and as such, ATP release may indeed represent a checkpoint to the immunogenicity of chemotherapy. Irrespective of these incognita, it appears clear that depletion of ATP from dying cells by inhibition of ATP synthesis or by addition of the ATP-degrading enzyme apyrase abolishes the immunogenicity of cancer cell death [84]. Indeed, ATP released from tumor cells acts on the purinergic P2RX7 receptor present on DC to facilitate anticancer immune responses (see below).

ATP and UTP can also play the role of non-redundant find-me signals that are released by apoptotic cells for their efficient clearance by monocytes/macrophages that express the purinergic receptor P2RY2 [86]. Forced expression of CD39 (NTPDase-1), an ecto-apyrase responsible for the degradation of NTP by immune cells in vivo [87] abrogated the chemoattractant activity of apoptotic cells [86], suggesting that tumor cells could control their clearance via the expression of CD39 or other enzymes that degrade ATP [88]. CD39 is overexpressed on some cancer cell types such as melanomas [89], and it will be interesting to correlated the expression of CD39 (and other ATP-degrading enzymes) with anticancer immune responses elicited by chemotherapy or radiotherapy.

4.8. Pro-tolerogenic factors: Gas6

The receptor tyrosine kinase Mer is involved in the phagocytosis of apoptotic cells by certain macrophage subpopulations [90]. The role of Mer in the immunoregulation of TLR signaling [91] and in the apoptosis-induced inactivation of CD11c+CD8α+ dendritic cells has been established [92]. Mutant mice lacking the three receptor tyrosine kinases TAM (Tyro3, Axl, Mer) show defective clearance of apoptotic bodies and develop severe lymphoproliferative disorders accompanied by broad spectrum autoimmunity [93]. The growth arrest specific gene 6 (Gas6) detectable on the surface of dying cells is a phosphatidylserine opsonin and a ligand for Mer (and Axl).
Blocking Gas6 prevents the inhibitory effects of apoptotic cells on CD11c⁺CD8α⁺ DC, restoring their activation and T cell stimulatory activity [92]. Axl/Gas6 signaling has been shown to regulate survival, proliferation and migration of a variety of tumor cell lines of epithelial, mesenchymal and hematopoietic origin, to be inducible by chemotherapy and to confer drug resistance [94,95]. Overexpression of Axl/Gas6 in renal cell carcinoma or glioblastoma or leukemia correlated with poor prognosis [96–98]. It remains to be established whether the TAM/Gas6 interaction affects clinical outcome through a tumor cell-autonomous pathway or rather through an effect on the dialogue between tumor cells and phagocytes.

5. Checkpoints for the immunogenicity of cell death at the host level

As outlined above, chemotherapy may fail because it is intrinsically unable to stimulate immunogenic cell death or because cells fail to emit the appropriate set of immunogenic signals as they die. In addition, chemotherapy may fail because the immune system is unable to perceive immunogenic signals or because it has been subjected to local or systemic immunosuppression.

5.1. The role of DC and T cells

If the host immune system plays a role in the antitumor effects mediated by cytotoxic agents, then, certain defects in genes encoding immune functions should subvert the clinical efficacy of these anticancer compounds. The first in vivo studies performed in 1973, comparing immunocompetent versus compromised mice, indicated that part of the antitumor activity of anthracyclines could be attributed to the host's immune system [99]. These findings were corroborated in various experimental models, and anthracyclines were shown to enhance innate and cognate immune functions in vivo [100,101]. Doxorubicin induces specific immune functions and cytokine expression in peritoneal cells [30,102]. However, for historical reasons, drug discovery programs for cancer therapy have overlooked the possibility that immune reactions might contribute to the success of treatment. Indeed, in 1976, the National Cancer Institute (NCI) edited guidelines for drug screening, prompting investigators to validate their strategy using human tumor cells xenotransplanted into immunodeficient mice [103].

Recently, we discovered that the oxaliplatin-mediated tumoricidal activity against EL-4 was completely abolished in mice deficient for the recombination activating protein 2 (Rag2, which lack both B and T cells), in athymic nu/nu mice (which lack T cells), and in wild type mice depleted from CD8⁺ lymphocytes [84]. Similarly, the antitumor efficacy of 10 Gy-irradiation against the breast cancer T/S/A was severely compromised in nu/nu mice [75]. Neutralizing antibodies directed against anti-CD4⁺ and CD8⁺ lymphocytes also abrogated the immune response against dying tumor cells [30]. Using CD11c-DTR transgenic mice in which diphertheria toxin depletes conventional DC, it was found that dendritic cells mobilized by doxorubicin-treated tumor cells are indispensable to elicit CTL responses that protect mice against rechallenge with live tumor cells [30]. Accordingly, tumor antigens derived from doxorubicin or oxaliplatin-treated cells can be cross-presented by host DC to MHC class I-restricted Tc1 lymphocytes [31,84]. Thus, cross-presentation of tumor antigens by DC may be decisive for dying cancer cells to elicit specific immune responses.

Since the mouse CD8α⁺ DC excels at cross-presenting antigens [104], Sancho et al. went on studying myeloid C type lectins uniquely expressed on this subset and their role in the immunogenicity of cell death. They showed that mouse CD8α⁺ DC take advantage of one of their surface molecules, DC/NK lectin group receptor-1 (DNGR-1, also called CLEC9A), to regulate cross-presentation of necrotic cells by signaling via SYK kinase [105]. The CLEC9A receptor handles dying cells resulting from secondary necrosis promoted through UV light, anthracyclines, freeze-thawing, or serum deprivation, but does not function in the phagocytosis of latex particles. Rather, the CLEC9A/SYK pathway may activate DC in response to dead cells, presumably in coordination with other danger receptors because targeting this DC receptor with antigen epitopes covalently coupled to a specific antibody requires adjuvant to elicit T cell priming [104]. Moreover, it is currently unknown whether CLEC9A contributes to antitumor immune responses.

5.2. The coordinated action of TLR4 and P2RX7

The systematic screening of the danger receptors such as Toll-like (TLR) and Nod-like receptors (NLR) revealed a major role for TLR4 and NLRP3 in the immunogenicity and efficacy of chemotherapy or radiotherapy in mice. Mutations in TLR4 that affect receptor signaling markedly decreased the efficacy of conventional anticancer therapies applied to a series of tumors growing on syngenic mice. This applies to X-rays used for the cure of established T/S/A mammary cancers, oxaliplatin employed against EL-4 thymoma and GOS osteosarcoma, as well as doxorubicin administered against CT26 colon cancers [75]. Accordingly, dying tumor cells failed to elicit antigen-specific Tc1 immune responses in TLR4−/− mice unless they were loaded onto bone marrow-derived DC bearing a TLR4 WT genotype. These results suggested that TLR4 must function within host DC for optimal efficacy of chemotherapy [75]. TLR4 signaling in DC involved Myd88 (but not TRIF) adaptor molecules and appeared to be critical for the dynamic of the endocytic compartments, the processing of the phagocytic cargo and the presentation of antigens by MHC class I molecules. TLR4 engagement by HMGB1 acted in coordination with the Nlpr3 inflammasome complex to induce the processing and maturation of IL-1β in DC [84]. Indeed, DC loaded with oxaliplatin-treated tumor cells secreted IL-1β in an Nlpr3−, ASC- and caspase-1-dependent fashion, and IL-1β secretion was blocked by neutralizing HMGB1 [84]. Therefore, the efficacy of doxorubicin or oxaliplatin against established tumors (whether transplantable or methycholanthrene-induced) was markedly impaired in animals bearing genetic defects in the Nlpr3→ ASC → Casp-1→ IL-1β→ IL-1R1 axis and mice treated with neutralizing anti-IL-1β antibodies (Fig. 3). In contrast, IL-1α and IL-1β did not contribute to the antitumor effects of these therapies [84].

Purinergic receptors P2RX7 expressed on DC were found to be strictly required for the activation of the Nlpr3 inflammasome and for the immune response against dying tumor cells [84]. P2RX7 receptors sense extracellular ATP. Removal of ATP from dying cells, scavenging of ATP by the ATP-degrading enzyme apyrase, or excess amounts of P2X receptor antagonists prevented the priming of tumor antigen-specific T cells by dying tumor cells and the prophylactic effects of vaccines composed of mitoxantrone or doxorubicin-treated CT26 against rechallenge with live cells [84].

Why is IL-1β produced by DC encountering dying tumor cells so critical for the efficacy of chemotherapy? The immune response ensuing in the draining lymph nodes of a tumor, 5–7 days post-exposure to X-rays or local doxorubicin or systemic oxaliplatin, requires tumor antigen-specific CD8⁺ T cells that produce IFN-γ in an ILR4- , caspase-1- and IL-1R1-dependent manner [84]. However, these IFN-γ producing T cells did not exhibit potent cytolytic activities against tumor cells. Accordingly, we found that signaling through IFN-γR and IFN-γ was mandatory for the efficacy of chemotherapy against a variety of different tumors, while IL-12Rβ2, perforin or TRAIL were dispensable [84]. Finally, IL-1β was shown to be a key cytokine gearing the polarization of TCR-triggered CD8⁺ (but not CD4⁺) T lymphocytes in vitro, yet lack-
ing the potential to modulate antigen processing or activation in DC.

Altogether, these results support the contention that two DAMP receptors, TLR4 (which senses HMGB1) and P2RX7 (which senses ATP), have to be activated in a concerted fashion to allow for anticancer immune responses to be efficient. Thus, a defined spatiotemporal pattern of cell death-associated DAMPs (CRT exposure, HMGB1 release, ATP release) functions like a “key” to open the “lock” that usually precludes an immune response, through the action of defined receptor present on the surface of DC.

6. Clinical data supporting the key/lock paradigm

A polymorphism in human TLR4 (rs4986790) resulting in a single-nucleotide exchange (896A/G) in the tlr4 gene and in an amino acid substitution (Asp299Gly) in the extracellular domain of TLR4 has been associated with decreased responses to inhaled lipopolysaccharide [106]. This substitution not only decreased the binding of HMGB1 to TLR4 but also resulted in a weaker activation of the transcription factor NF-κB ([107] and unpublished data) as well as in a profound alteration of the capacity of monocyte derived-human DC to cross-present melanoma tumor antigens from dying melanoma cell lines [75]. In a retrospective study, we analyzed the time to metastatic progression in a cohort of 280 patients that had been treated for non-metastatic breast cancer with local lymph node invasion, following a standard protocol of local surgery, local radiotherapy and systemic anthracycline injections (FEC protocol). Patients carrying TLR4 Asp299Gly allele (about 17%) did not differ from patients displaying the normal TLR4 allele for all classical prognostic factors. However, patients bearing the TLR4 Asp299Gly allele developed metastasis more rapidly than patients bearing the normal TLR4 allele, establishing TLR4 Asp299Gly as an independent predictive factor of early disease progression [75].

Next, we investigated whether the same loss-of-function allele of tlr4 could affect the progression-free survival (PFS) of metastatic colorectal cancer (CRC) patients (n=338) undergoing an oxaliplatin-based regimen. Patients that were heterozygous or homozygous for the tlr4 Asp299Gly/Thr399Ile allele (n=48) did not differ from patients bearing the normal TLR4 allele (n=290) with respect to prognostic parameters relevant in CRC. Once again, patients bearing the normal TLR4 allele manifested an increased PFS (Hazard ratio 0.73, CI [0.53; 1.00], p < 0.05) and overall survival (OS) (Hazard ratio 0.72, CI [0.52; 1.01], p = 0.05), as compared to patients bearing the loss-of-function allele of TLR4. In contrast, in a cohort of stage II CRC patients (n=258) who were treated with surgical removal of the primary tumor in a curative intent, without any adjuvant chemotherapy, no statistical differences in the terms of disease-free survival among patients bearing the normal or variant allele of tlr4. This result suggests that tlr4Asp299Gly is not a prognostic factor but rather a predictive factor of the response to oxaliplatin [108].

More recently, we investigated the prognostic value of a single-nucleotide polymorphism in the ligand-gated channel P2RX7 at nucleotide position 1513 (1513A>C) changing a glutamic acid to alanine at aa 496 (Glu496Ala) which abrogates the ATP-induced Ca2+ and ethidium influx (and the K+ efflux) and severely retards the ATP-dependent IL-1β release from monocytes [109]. We analyzed a cohort of 225 sporadic breast cancer patients that were stratified according to the P2RX7 genotype (normal (64%) versus variant (36%) P2RX7). While there was no significant differences in classical prognostic factors between the normal and variant groups of patients, the P2RX7 loss-of-function allele had a significant negative prognostic impact on metastatic disease-free survival (Log rank test; p = 0.02). A multivariate Cox regression model revealed a significant effect, both for the tumor grade and for the P2RX7 genotype.

Altogether, these data suggest that selective immune defects (in the DC-mediated presentation of antigen from dying cells or in IL-1β release) can compromise the response to anticancer radiotherapy and chemotherapy, at least in node positive (N+) breast cancers treated with adjuvant anthracyclines. However, it is noteworthy that in vitro studies indicated that homozygous loss-of-function of P2RX7 are accompanied with a marked defect in IL-1β release and that P2RX7 also initiates downstream events such as the stimulation of a metalloproteinase causing the shedding of 1-selectin from monocytes and lymphocytes [109]. Therefore, prospective long-term studies correlating immune functions with loss-of-function SNPs and time to progression are needed.

7. Immunological prospects for personalized chemotherapy

7.1. Promoting the immune response following tumor cell death

Several strategies have been attempted in preclinical studies that have been reviewed elsewhere [110,111]. Some recent findings will be reported below.
Inducing cell death by targeting TRAIL receptors may be a reasonable strategy not only to bypass tumor resistance to mitochondrial membrane permeabilizing agents, but also to generate an immunogenic cell death pathway [112]. When comparing the immunogenicity of B16F10 killed by a specific TRAIL-expressing DC cell subpopulation versus perforin/Granzyme B expressing NK cells, we found that tumor cells are particularly effective in eliciting prophylactic antennatumor activity [113]. Accordingly, TRAIL can stimulate CRT exposure on tumor cells [32]. Low dose cyclophosphamide may induce TRAIL expression on T and/or NK effectors and promote the eradication of TRAIL-sensitive tumors [114]. When combined with TLR2/4 agonists, cyclophosphamide induced TRAIL expression on DC and DC became tumor killers leading to antigen cross-presentation and T cell and TRAIL-dependent antennatumor effects [115]. Finally, antibodies targeting not only DR5 but also costimulatory molecules expressed by DC (such as anti-CD40 or anti-CD1d Ab) and T cells (such as anti-CD137 mAb) mediated potent synergistic antennatumor effects against TRAIL-sensitive tumors [116]. In TRAIL-resistant tumors, the combination of doxorubicin or gemcitabine with anti-CD1d and anti-CD137 agonistic Ab improved antennatumor activity [117]. Finally, it is feasible to improve targeted therapies of ErbB-2/HER2+ breast cancer by using a combination of anti-DR5 and anti-ErbB-2 antibodies, which both significantly suppressed the growth of advanced spontaneous tumors arising in ErbB-2-neuT transgenic mice, in a CD11b+ and CD8+ T cell-dependent manner [118].

In genetically engineered Hgf-Cdk4R24C mice where sporadic melanomas develop, complete cure of primary and metastatic disease could only be achieved by a combination of four strategies, i.e. (i) chemotherapy (alkylating agents), (ii) the adoptive transfer of p-mel specific CD8+ T cells, (iii) adenoviral vectors engineered to express the gp100 antigen and (iv) immunostimulatory nucleic acids in the tumor microenvironment, all culminating in expansion, differentiation and survival of IFN-γ producing CTLs, sparing healthy tissues [119].

Neutralizing immunosuppressive pathways together with chemotherapy has also been successful. Combining anti-CTLA4 with a conditioning regimen for allogeneic hematopoietic stem cell transplantation proved safe and efficient for 3/29 patients in relapse, without causing overt graft-versus-host disease [120]. Combining anti-PD-1 Ab with gemcitabine was synergistic in a mouse model of pancreatic cancer [121]. Chemoinmunotherapy associating chemotherapy (paclitaxel, gemcitabine or cyclophosphamide) with the D stereoisomer of 1-methyl-tryptophan (inhibitors of indoleamine 2,3-dioxygenase) was more efficacious than each agent alone [122]. Promoting the exhaustion and apoptosis of intratumoral Treg using a combination of cyclophosphamide and agonistic anti-OX40 Ab may result in potent synergistic antitumor effects against B16F10 melanoma [123].

These examples illustrate the possibility to combine therapies that induce immunogenic cell death with immunostimulatory regimes to mediate an “immunochemotherapeutic” synergy.

7.2. Compensating defects at the level of the tumor and of the host

Pinpointing the molecular defects at the level of the tumor might result in a specific therapeutic intervention. Thus, restoring the capacity of a tumor to expose CRT can be achieved by manipulating the PERK → eIF2α axis and the PP1-GADD34 complex using specific ER stress response modifiers or specific inhibitory compounds, respectively [32,33]. Local injection of recombinant CRT into tumors that lack essential compounds of the CRT translocation machinery (such as Erp57) can also re-establish the sensitivity to chemotherapy [59].

Identifying the immunological defects at the level of the host may also facilitate a targeted compensation (Fig. 4). This has been achieved by using chloroquine in mice deficient for TLR4 [75] or by coadministering TLR3 or TLR9 agonists [107]. In the former case, the lysosomotropic chloroquine given at the time of cell death may restore the dynamics of the endocytic compartments in DC, thereby favoring antigen cross-presentation and T cell priming [75]. In the latter case, TLR3 or TLR9 likewise stimulate the Myd88 pathway that fails to be activated by deficient TLR4. In mice that lacked P2RX7, elements of the Nlrp3 inflammasome (Nlrp3, caspase-1) or IL-1β, we could show that exogenous recombinant IL-1β or rIL-12 restored the Tc1 immune response triggered by cell death inducers [84]. However, exogenous IL-1β was unable to restore the failing anticancer immune response in mice lacking TLR4. These results underscore the importance of diagnosing immune defects in order to proceed to a case-specific therapeutic restoration of the immune response.

8. Concluding remarks

An ideal vaccination strategy against tumors should rely on specific antigens required for tumor maintenance, such as those involved in the oncogenic process or the autoantigens dictating cell cycle regulation or cell stress. Strong preclinical and clinical evidence supports that X ray-induced death of tumor cells genetically modified to express GM-CSF do mount humoral and cellular immune responses while in parallel, the contribution of the host...
immune system in the efficacy of some chemotherapies is being demonstrated. In all cases, the clinical success remains limited or the “immunochemotherapy” approach is adequate or suitable for a limited subset of tumors and patients that remains to be identified based on the molecular dialogue between dying tumor cells and immune effectors.

Prospective translational studies are required to elucidate which among the theoretical checkpoints dictating the immunogenic cell death and residing at the level of the tumor, of the drug and of the host will prevail in controlling humoral and cellular immune responses, as well as the clinical outcome. We anticipate that the validation of at least some of these checkpoints will allow (i) to device algorithms predictive of clinical responses, (ii) to personalize therapy with cell death inducers, and (iii) to re-orient immunization strategies according the pre-existing immune status of the patients (Fig. 5).

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