Cell Death and Differentiation (2014) 21, 15–25

www.nature.com/cdd

Review

Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer

L Bracci*[,1], G Schiavoni[1], A Sistigu[1] and F Belardelli[1]

Conventional anticancer chemotherapy has been historically thought to act through direct killing of tumor cells. This concept stems from the fact that cytotoxic drugs interfere with DNA synthesis and replication. Accumulating evidence, however, indicates that the antitumor activities of chemotherapy also rely on several off-target effects, especially directed to the host immune system, that cooperate for successful tumor eradication. Chemotherapeutic agents stimulate both the innate and adaptive arms of the immune system through several modalities: (i) by promoting specific rearrangements on dying tumor cells, which render them visible to the immune system; (ii) by influencing the homeostasis of the hematopoietic compartment through transient lymphodepletion followed by rebound replenishment of immune cell pools; (iii) by subverting tumor-induced immunosuppressive mechanisms and (iv) by exerting direct or indirect stimulatory effects on immune effectors. Among the indirect ways of immune cell stimulation, some cytotoxic drugs have been shown to induce an immunogenic type of cell death in tumor cells, resulting in the emission of specific signals that trigger phagocytosis of cell debris and promote the maturation of dendritic cells, ultimately resulting in the induction of potent antitumor responses. Here, we provide an extensive overview of the multiple immune-based mechanisms exploited by the most commonly employed cytotoxic drugs, with the final aim of identifying prerequisites for optimal combination with immunotherapy strategies for the development of more effective treatments against cancer.
Cell Death and Differentiation (2014) **21**, 15–25; doi:10.1038/cdd.2013.67; published online 21 June 2013

Facts

(1) Immune competence is crucially required for chemotherapy efficacy.
(2) The antitumor effects of chemotherapy rely on tumor–host interplay.
(3) Tumor-induced immune tolerance needs to be overcome in order to allow the full recovery of immune surveillance and hamper tumor spreading or recurrence.
(4) The capability of some cytotoxic drugs to induce an immunogenic cell death makes apoptotic tumor cells a good endogenous vaccine.
(5) Some cytotoxic agents induce a lymphodepletion-associated bystander immune stimulation.

Open Questions

(1) Patient's immune status and tumor cells' intrinsic characteristics need to be investigated deeper, so as to identify the requisites predicting full benefit of defined single or combined anticancer treatments.
(2) Off-target effects of chemotherapy need further elucidation in order to allow a rationale-based, rather than empirical, selection of combinatorial regimens.
(3) Adjuvant chemotherapy regimens using cytotoxic drugs with immunomodulatory properties, possibly in combination with immunotherapy approaches, should be evaluated as strategies for tertiary prevention of cancer.
(4) Strategies to be pursued should be aimed at selectively hitting tumor-induced inhibitory cells/mechanisms, sparing effector cells and other subsets capable of suppressing undesirable autoimmune responses.

Conventional anticancer chemotherapy is generally thought to act through selective killing of tumor cells or by irreversibly arresting their growth. Cytotoxic drugs interfere with DNA synthesis, or produce chemical damage to DNA, ultimately leading to tumor cell death (TCD; see Table 1). However, this concept neglects the possible contribution of the host to the therapeutic process of chemotherapy. Accumulating evidence indicates that several chemotherapeutic drugs are more efficient against tumors that are implanted in immunocompetent, with respect to immunodeficient, hosts.[1]

[1]Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy
*Corresponding author: L Bracci, Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanitã, viale Regina Elena 299, Rome 00161, Italy. Tel: +39 06 4990 2474; Fax: +39 06 4990 2714; E-mail: laura.bracci@iss.it
Keywords: chemotherapy; cancer; immune system; cellular immunity; combined therapies
Abbreviations: DC, dendritic cell; NK, natural killer; TAM, tumor-associated macrophages; M1, classically activated macrophages; M2, alternatively activated macrophages; Treg, regulatory T; MDSC, myeloid-derived suppressor cell; CTX, cyclophosphamide; 5-FU, 5-Fluorouracil; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TCD, tumor cell death; BM, bone marrow; LN, lymph node; HSPs, heat shock proteins; VEGF, vascular endothelial growth factor; HMGB1, high-mobility group box-1; ecto-CRT, surface-exposed calreticulin; TK, tyrosine kinase; mTOR, mammalian target of rapamycin
Received 11.2.13; revised 07.5.13; accepted 14.5.13; Edited by M Piacentini; published online 21.6.13

Table 1 Biochemical and biological properties of conventional antineoplastic drugs

Class	Examples	Biochemical activity	Biological effects	Use in clinical oncology
Antimetabolites	5-Fluorouracil	Analog of pyrimidine nucleoside	Perturbation of RNA and DNA synthesis	Colorectal cancer, pancreatic cancer
	Gemcitabine			Non-small cell lung cancer, pancreatic cancer, bladder cancer, breast cancer
	Methotrexate	Inhibits dihydrofolate reductase	Reduction of folates required for DNA synthesis	Breast, head and neck, leukemia, lymphoma, lung, osteosarcoma, bladder and trophoblastic neoplasms
Alkylating agents	Cyclophosphamide	Adds an alkyl group to DNA	Inhibition of DNA replication	Lymphomas, leukemias, brain tumors
	Dacarbazine			Metastatic melanoma, Hodgkin's lymphoma
	Melphalan			Multiple myeloma, ovarian cancer, malignant melanoma
Anthracyclines	Doxorubicin	Intercalates base pairs of nucleic acids	Inhibition of RNA and DNA synthesis	Leukemias, Hodgkin's lymphoma, bladder cancer, breast, stomach, lung, ovaries, thyroid, soft-tissue sarcoma, multiple myeloma
Antimicrotubule agents	Vinblastine	Binds tubulin, thereby inhibiting the assembly of microtubules	M-phase-specific cell cycle arrest by disrupting microtubule assembly	Hodgkin's lymphoma, non-small cell lung cancer, breast cancer, head and neck cancer, and testicular cancer
Platinum compounds	Cisplatin	Crosslinks DNA strands	Inhibition of DNA replication and transcription	Head and neck, lung and ovarian carcinomas, lymphomas, germ cell tumors
	Oxaliplatin			Colorectal cancer
Taxanes	Paclitaxel	Stabilizes GDP-bound tubulin in microtubules	Inhibition of mitosis	Lung, ovarian, breast, head and neck cancer, advanced forms of Kaposi's sarcoma
	Docetaxel			Breast, ovarian, prostate and non-small cell lung cancer
Topoisomerase inhibitors	Irinotecan	Interferes with type I topoisomerases inducing DNA strands breaks	Cell cycle arrest and apoptosis	Colorectal cancer
	Etoposide phosphate	Interferes with type II topoisomerases inducing DNA strands breaks		Kaposi's and Ewing's sarcomas, certain leukemias, lung, ovarian, gastrointestinal cancers, glioblastoma multiforme
	Mitoxantrone			Metastatic breast cancer, acute myeloid leukemia and non-Hodgkin's lymphoma

Conventional chemotherapy can stimulate the immune system in two ways. Some agents elicit cellular rearrangements that render dying tumor cells visible to the immune system. Other drugs induce a transient lymphodepletion, subvert immunosuppressive mechanisms, or exert direct or indirect stimulatory effects on immune effectors. Immunomodulatory properties are being ascribed also to targeted chemotherapeutic agents, that is, compounds designed to hit biochemical pathways essentially required for tumor cell survival and/or growth (Box 1). All these observations open to the intriguing possibility that immunomodulatory chemotherapeutic agents may be good candidates for combination with immune-based therapeutic approaches.

This review will provide an overview on the immune-based mechanisms exploited by some cytotoxic drugs (Figure 1), with the final aim of identifying prerequisites for optimal combination with immunotherapy strategies for the development of more effective, rationale-driven treatments against cancer.

Effects on the Innate Immune System

Several direct effects of cytotoxic drugs have been described for macrophages, dendritic cells (DCs) and natural killer (NK) cells. Earlier studies on NK-cell function in cancer patients undergoing cytotoxic chemotherapy have shown variable effects, especially in correlation with the clinical outcome.[2,3] In breast cancer patients with localized and metastatic disease, cytotoxic drug regimens were shown to induce an overall impairment of NK-cell responses.[4,5] Recently, a metronomic (that is, low dosage for a prolonged period of time) cyclophosphamide (CTX) regimen was shown to potently stimulate NK-dependent antitumor immunity in end-stage cancer patients[6] and the prompt recruitment of DCs, macrophages and NK cells to the tumor site in diverse mouse models.[7,8] Interestingly, combined treatment with 5-Fluorouracil (5-FU) and IFN-α resulted in higher numbers of infiltrating NK cells with enhanced cytotoxicity in a pancreatic tumor model.[9]

Effects of chemotherapy on macrophages have also been documented. Macrophages can differentiate from blood monocytes into two distinct subtypes, namely classically activated (M1) and alternatively activated (M2) macrophages endowed with effector or suppressive functions, respectively.[10,11] Macrophages infiltrating solid tumors (that is, tumor-associated macrophages or TAMs) share many characteristics with M2 macrophages and exert a pro-tumorigenic function in virtue of their direct or indirect (via cytokine production) immune-suppressive effects towards NK and T-cells.[12] In cancer patients, the presence of TAMs favors tumor progression.[12] Several studies have investigated the

Box 1 Immune-based effects of targeted anticancer compounds

Targeted therapies act by blocking biochemical pathways or mutant proteins essentially required for tumor cell growth and survival. Most targeted therapies induce dramatic tumor regressions, although long-term clinical benefit is hampered by the occurrence of drug-resistant variants. Similar to conventional chemotherapeutic agents, some targeted agents display immunomodulatory properties. The receptor tyrosine kinase (TK) inhibitor sunitinib dampens the immunosuppressive activity of Treg and MDSCs,[133,134] whereas the TK inhibitor imatinib challenges IDO expression in myeloid cells.[135] mTOR kinase inhibitors (e.g., everolimus, temsirolimus) trigger autophagy and inhibit angiogenic activity by both direct effects on vascular cell proliferation and indirect effects on growth factor production.[106] Diverse Janus kinase inhibitors, small molecules interfering with cytokine signaling, prove effective in stimulating DC maturation and antigen-specific T-cell priming.[136] The proteasome inhibitor Bortezomib, which is approved for use in multiple myeloma, sensitizes tumor cells to TRAIL- and NK-mediated cell lysis.[137] Vemurafenib, a specific BRAF inhibitor recently approved for treatment of some melanomas, enhances the expression of several tumor antigens, enabling immune recognition by T lymphocytes.[138] Trastuzumab and cetuximab, monoclonal antibodies directed against tumor-associated receptor TK HER-2 and EGFR, respectively, augment antigen presentation through the formation of immune complexes, leading to stimulation of T-cell-mediated immune responses.[139,140] Altogether, these observations pave the way to clinical studies aimed at evaluating the possible synergism of targeted compounds and immunotherapy strategies.

effects of cytotoxic chemotherapy in subverting the protumorigenic activities of macrophages. For example, low-dose CTX can promote the skewing of M2 macrophages into M1 *in vivo,* thus enhancing the production of oxygen radicals, IL-6 and IL-12, and potentiating innate responses.[13] Similarly, in mice bearing B16.F10 melanoma, combined treatment with vincristine, CTX and doxorubicin resulted in substantial enrichment of a TAM subpopulation that can be M1-polarized upon concomitant immunotherapy.[14] Interestingly, whereas tumor sensitivity to CTX or cisplatin *in vitro* is increased when tumor cells are cultivated with macrophages, coculture of macrophages with human primary ovarian tumor cells decreased tumor sensitivity to 5-FU.[15] Likewise, the taxane paclitaxel can stimulate TAMs cytotoxicity directly[16] and induce the activation of DCs, NK and tumor-specific CTL via the secretion of IL-12 and TNF-α and inducible nitric oxide synthase (iNOS) by TAMs,[17] resulting in tumor regression. Conversely, paclitaxel-induced influx of TAMs was detrimental to chemotherapy response in mouse mammary carcinoma and breast cancer patients.[18,19]

Chemotherapy has influence on bone marrow (BM) hematopoiesis, affecting myeloid cell mobilization differentially. A single injection of low-dose CTX was shown to spare DC precursors in the BM, promoting their expansion and differentiation in the peripheral lymphoid organs. CTX exerted its effects preferentially on the CD8α-expressing-DC subset, determining an initial ablation of lymphoid organ-resident CD8α^+ DCs, followed by overshoot replenishment after drug discontinuation.[20,21] Data from our laboratory have revealed that, after CTX administration, CD8α^+ DCs migrate to the tumor site where they cross-present tumor-associated antigen (Figure 2). The platinum-based compound cisplatin was also reported to modulate the percentages of myeloid cells by increasing DCs and eliminating myeloid-derived suppressor cells (MDSCs), thus favoring immune effector responses in melanoma-bearing mice.[22] Moreover, an overall increase of CD14$^+$ monocytes, CD11c$^+$ myeloid DCs and CD123$^+$ plasmacytoid DCs was observed in patients with advanced pancreatic cancer receiving gemcitabine for 2 months, with respect to untreated patients.[23]

Direct immunostimulatory effects of cytotoxic drugs on DC activities were also reported. An unbiased functional screen of 54 chemotherapeutic agents unveiled striking diversity of the tested drugs on the maturation, survival and growth of DCs.[24] The drugs delivering DC maturation signals at concentrations causing only marginal DC death included topoisomerase inhibitors (for example, etoposide, mitoxantrone, doxorubicin), antimicrotubule agents (for example, vinblastine, paclitaxel, docetaxel) and the two alkylating agents mechlorethamine and diaziquone.[24] In another report, paclitaxel, doxorubicin and methotrexate were shown to promote the ability of murine BM–DCs to present antigens to T-cells *in vitro* by upregulating antigen-processing machinery gene components, costimulatory molecules and IL-12p70.[25] Similar results were observed with human monocyte-derived DCs.[26] Notably, vinblastine at low concentrations (0.1–1 μM) induces phenotypic and functional maturation of DCs *in vitro* and *in vivo*, when injected into the skin of mice, by triggering *in situ* maturation of skin-resident DCs and by boosting humoral and cellular immune responses.[27] A recent study on human 6-sulfo LacNAc$^+$ (slan) DCs, a major subpopulation of human blood DCs, showed that doxorubicin impairs the ability of these cells to produce proinflammatory cytokines and to activate T lymphocytes and NK cells, whereas methotrexate and paclitaxel sustain their effector properties.[28]

Cytotoxic chemotherapy can affect DC activities also through indirect mechanisms. Pioneering studies showed that 5-FU and doxorubicin induced *in vitro* cancer expression of heat shock proteins (HSPs) that promote the engulfment of cell debris by human DCs and the subsequent cross-presentation of tumor antigens to T-cells.[29,30] More recently, Casares *et al.*[31] showed that doxorubicin-killed tumor cells elicit tumor-specific immune responses when injected into syngeneic mice, by stimulating DC phagocytosis and CD8 T-cell responses. Subsequent work from L. Zitvogel's laboratory identified the molecular mechanism linking immunogenic apoptosis induced by chemotherapy to DC activation, as detailed below.

Effects on the Adaptive Immune System

Treatment of cancer patients with intensive chemotherapy results in profound depletion of all lymphocytic populations, especially of B cells.[32,33] A study conducted on breast cancer patients to evaluate the effect of combination chemotherapy regimens with epirubicin (5-FU, epirubicin, CTX) *versus* doxorubicin (5-FU, doxorubicin, CTX) on subsets of immune cells revealed an increase in the percentages of cytotoxic T and NK cells, and a dramatic decrease in that of B cells in the blood following either regimen.[34] Similarly, repeated gemcitabine cycles reduced B cell frequencies and induced a profound suppression of antigen-specific IgG antibody

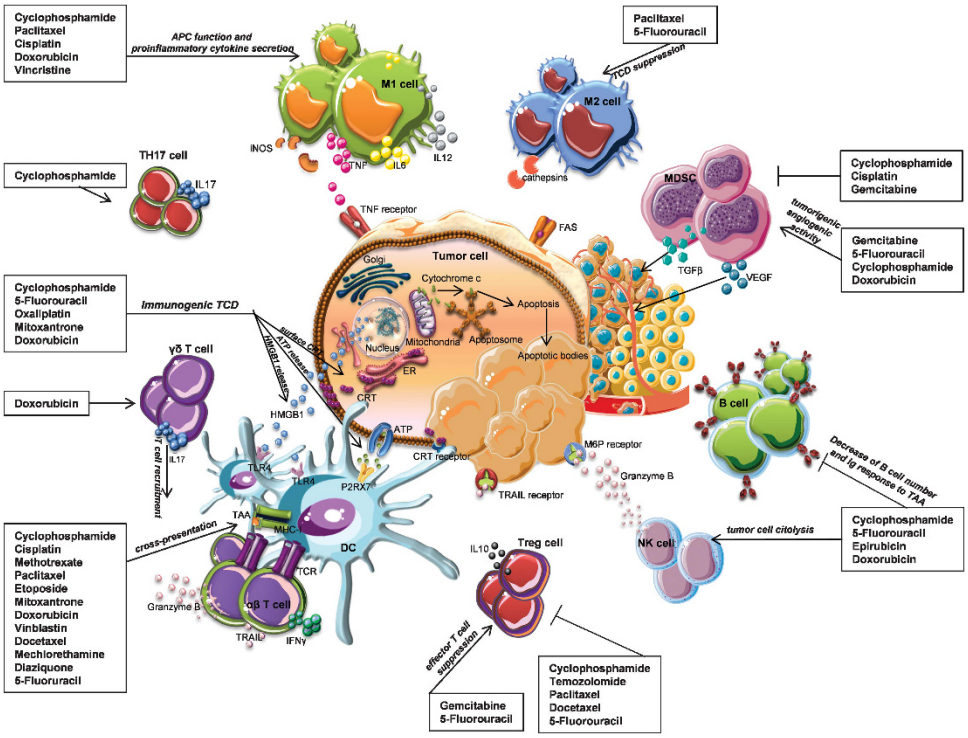

Figure 1 Immunomodulation by conventional cytotoxic drugs. Conventional antineoplastic drugs can activate anticancer immune responses through different mechanisms: (i) the inhibition of tumor-induced-suppressive mechanisms, (ii) the direct stimulation of T and B cell responses, (iii) the enhancement of tumor immunovisibility by cytotoxic cell subsets or phagocytes. Low-dose CTX and gemcitabine deplete regulatory T-cells or myeloid suppressor cells and facilitate tumor attack by effectors. Paclitaxel, cisplatin and doxorubicin induce the upregulation of mannose-6-phosphate receptors on the surface of tumor cells, rendering them permeable to granzyme B. Paclitaxel induces proinflammatory cytokines' secretion from macrophages, leading to DC, NK and T-cell activation. Anthracyclines, oxaliplatin and CTX promote tumor expression of ecto-CRT, and release of HMGB1 and ATP by dying tumor cells, thus stimulating antigen phagocytosis and cross-presentation by DC

responses, but enhanced T-cell responses, in a mouse model of malignant mesothelioma.[35] The reason for this differential effect on the two lymphocytic populations was partly attributed to an increased sensitivity of B cells to the antiproliferative effects of gemcitabine *in vitro*, with respect to T-cells,[35] although it is not clear whether this mechanism also applies *in vivo*, or in the case of other chemotherapeutic agents. Instead, gemcitabine given at a single dose (120 mg/kg) preserved both T and B lymphocytes in the spleens of animals bearing large tumors,[36] and 5-FU-based adjuvant chemotherapy induced prominent tumor-specific antibody responses in colon cancer patients.[37]

The effects of CTX on humoral responses appear controversial. In some reports, CTX, even at low-dose regimens, exerted suppressive effects on humoral responses while boosting cellular responses, suggesting that B cells are particularly sensitive to CTX-induced cytotoxic effects.[38,39] In other reports, low-dose CTX was shown to increase the relative percentages of B and T-cells in mice bearing SW1C melanoma,[7] and the cellular and antibody responses in patients with advanced cancer.[40] Subsequent studies showed that the lymphodepleting effects induced by CTX are transient and that, soon after drug discontinuation, a homeostatic rebound overshoot of the lymphocytic pool occurs.[41,42] This implies that after drug administration, a reduction of both humoral and cellular responses may occur, but with different timing and kinetics. It has been proposed that the homeostatic replenishment of B and T lymphocyte compartments is sustained by a drug-induced 'cytokine storm' during which several hematopoietic and homeostatic factors, danger signals, pattern recognition receptors, inflammatory mediators and growth factors are expressed, thus boosting peripheral expansion.[41,43] One might speculate that these mediators cooperate in sensing and amplifying drug-induced myelo- and lymphotoxicity, thus stimulating a DNA damage response that, in turn, promotes immune activation. Interestingly, genotoxic stress activates the expression of IFN-α and IFN-λ genes, leading to the ultimate stimulation of immune responses resembling those evoked during viral infections.[44] In addition, CTX and fludarabine combination greatly improved the therapeutic efficacy of adoptively transferred tumor-specific B cells in a mouse melanoma model of experimental metastasis.[45] Likewise, combined high-dose CTX and doxorubicin treatment augmented long-lasting humoral response *in vivo* to a cancer vaccine.[46]

Numerous evidence indicate the benefits of chemotherapy on T-cell-mediated immune responses. Mice vaccinated with doxorubicin- or cisplatin-treated ovarian cancer cells have enhanced antitumor immunity, and prolonged survival largely dependent on CD4 T-cell-mediated immune responses.[47] Low-dose cisplatin and paclitaxel synergize to generate strong tumor-specific CD8 T-cell responses, through IL-2

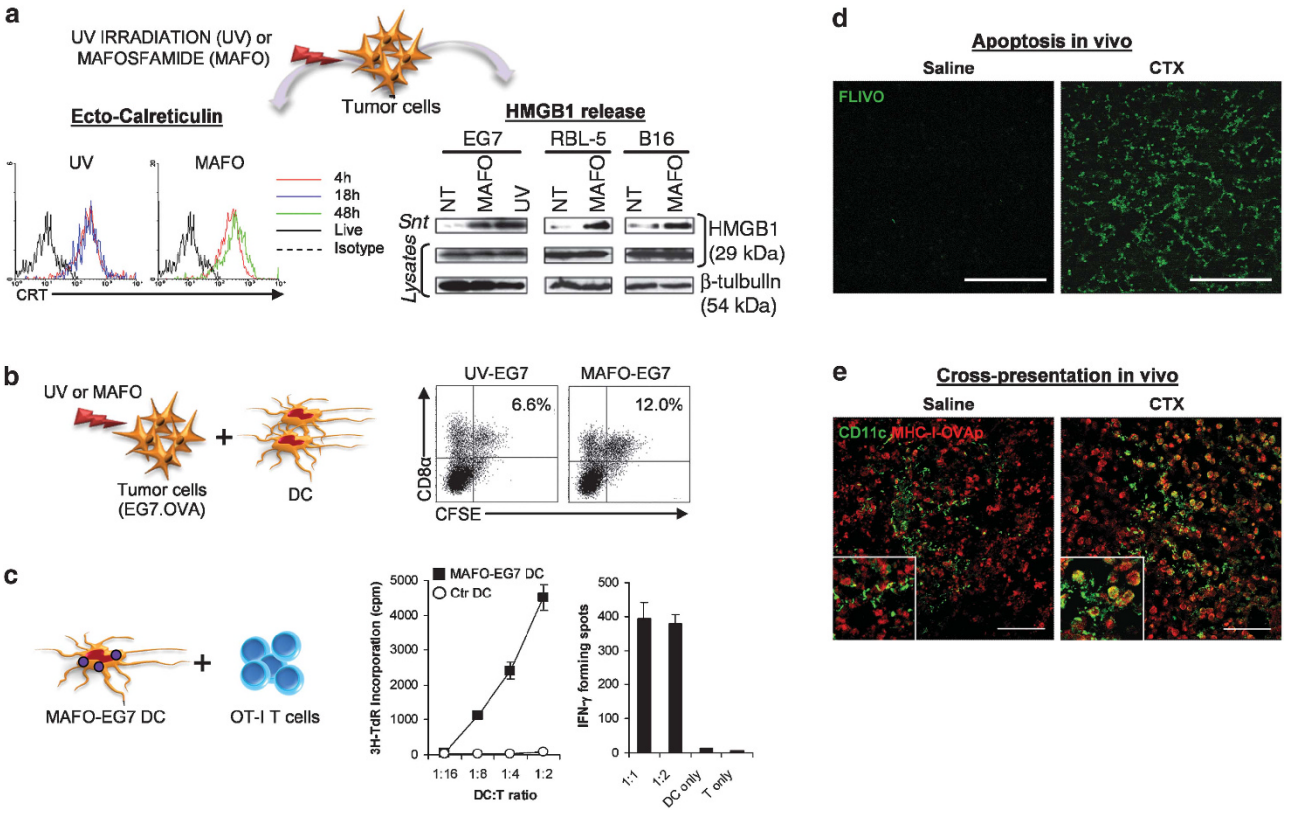

Figure 2 Induction of immunogenic tumor cell death and stimulation of DC cross-presentation by CTX *in vitro* and *in vivo*. (**a**) Ecto-CRT exposure at 4 h and extracellular HMGB1 release (48 h) in tumor cells following UV irradiation or treatment with the CTX-analog Mafosfamide (MAFO). (**b**) *In vitro* uptake of CFSE-labeled OVA-expressing EG7 tumor cells, killed by UV irradiation or MAFO treatment, by splenic CD8α + DC. (**c**) Stimulation of OT-I CD8 T-cell cross-priming by CD8α + DC that had captured MAFO-killed EG7 tumor cells. Proliferative response (left) and IFN-γ ELISPOT assay (right) are shown. (**d**) Induction of tumor apoptosis in EG7 tumor-bearing mice 2 days after a single injection of CTX (100 mg/kg), as detected by FLIVO staining in the tumor tissue. (**e**) *In situ* cross-presentation of tumor-associated antigen by tumor-infiltrating DC in mice bearing EG7 tumors following CTX injection (7 days). Co-expression of CD11c and OVA-derived peptide SIINFEKL bound to MHC class I (MHC-I-OVAp) in EG7 tumor tissue sections were detected by confocal laser-scanning microscopy. Modified from Schiavoni *et al*[21]

and IFN-γ secretion, and high therapeutic efficacy on platinum-resistant ovarian cancers in both mice and patients.[48] 5-FU was also reported to increase IFN-γ production by tumor-specific CD8 T-cells infiltrating the tumor, and to boost T-cell-dependent antitumor responses by *in vivo* elimination of MDSCs.[49] In both mouse models and patients with esophageal squamous cell carcinoma, neoadjuvant chemotherapy with 5-FU and cisplatin increased the intratumoral trafficking of CD4 and CD8 T-cells.[50,51] In experimental carcinogen-induced adenocarcinomas and fibrosarcomas, doxorubicin treatment enhanced tumor-specific proliferation of CD8 T-cells in tumor-draining lymph nodes (LNs) and promoted tumor infiltration of activated, IFN-γ-producing CD8 T-cells.[52] In this setting, therapeutic efficacy of doxorubicin required both IL-1β and IL-17, and the presence of $\gamma\delta$ T-cells.

A single CTX injection potently enhances the antitumor response of tumor-bearing mice following adoptive transfer of tumor-reactive T-cells.[42,53,54] Several mechanisms have been proposed to explain this effect: (i) drug-induced bystander proliferation of memory CD4 and CD8 T-cells;[55] (ii) drug-induced stimulation of CD8 T-cells proliferation and IFN-γ production;[41] (iii) differentiation of adoptively transferred antigen-specific CD4 T-cells into activated polyfunctional T helper cells with potent antitumor activity;[56] (iv) specific homing of transferred T-cells to the lymphoid organs and tumor mass soon after drug discontinuation.[41,57] Accordingly, the therapeutic efficacy of combined CTX and adoptive transfer of tumor-specific spleen cells was shown to critically require donor CD4 T-cells.[42] Combined treatment with anti-4-1BB and CTX produced synergistic CD8-mediated anticancer effects in the B16 melanoma mouse model.[58]

Evidence for the positive impact of chemotherapy on antitumor immune responses also arises from pilot clinical trials with cancer vaccines. Gene expression analysis of peripheral blood mononuclear cells (PBMCs) from melanoma patients treated with dacarbazine and a peptide-based vaccine revealed, by 1 day after chemotherapy, increased expression of immunoregulatory factors that can account for the enhancement of tumor antigen-specific CD8 T-cell responses observed in those patients, compared with patients treated with vaccine alone.[59] These effects were paralleled by a widening of the antigenic repertoire and by an expansion of antigen-specific T-cell tumor reactivity.[60]

Cancer often results in an imbalance of Th1/Th2 immunity, which can be restored by some antineoplastic drugs. As an example, paclitaxel augments Th1 cellular immunity in patients with advanced non-small cell lung cancer by increasing the levels of circulating IFN-γ-secreting CD8

T-cells and IL-2-secreting CD4 T-cells.[61] Moreover, CTX induces Th1-polarizing cytokines (IL-2 and IFN-γ) and decreases Th2 cytokines (IL-4 and IL-10) both in tumor-bearing mice[41] and rats.[62] Notably, in tumor-bearing mice CTX increased the frequencies of splenic Th1 and Th17 cells, which displayed a faster recovery from drug-induced lymphodepletion, with respect to Treg cells.[43]

Th17 cells are a T-cell subset having important roles in inflammatory and autoimmune diseases.[63] However, their role in tumor pathogenesis and treatment remains controversial.[64,65] Recently, gemcitabine and 5-FU were shown to activate the inflammasome pathway in MDSCs, leading to IL-1β production, which, in turn, induced IL-17 secretion by CD4 T-cells and blunted the anticancer efficacy of chemotherapy.[66] In another report, however, optimal anticancer responses during doxorubicin treatment have been shown to require the IL-17-producing $\gamma\delta$ T-cell population.[52] A dose-dependent effect on the expansion or differentiation of CD4 T-cells producing IL-17A was also observed in naive and tumor-bearing mice following treatment with CTX.[67] In the same report, it was shown that the levels of IL-17 secretion in PBMCs after T-cell receptor stimulation were significantly enhanced in patients treated with metronomic CTX, with CD4 T-cells being the major source of IL-17.[67] Nevertheless, whether CTX-induced Th17 contributes to the antitumor efficacy of CTX remains unclear.

Effects on Regulatory Subsets and Pathways

Besides the active stimulation of effector cells, immunopotentiation by cytotoxic chemotherapy can also be achieved through the inhibition of tumor-induced immune suppression. Several subsets of immunoregulatory cells have been identified so far in cancer patients.[68] CD4-CD25-expressing Tregs and myeloid cells with suppressive functions, namely MDSCs and TAMs, accumulate in the blood and, especially, within tumor burden, thus contributing to disease progression through various mechanisms.[68,69]

Different strategies to achieve therapeutic depletion of suppressive cell subsets have been described so far.[70–72] Gemcitabine kills MDSCs, both *in vitro* and *in vivo*,[49,73] with no significant reduction in other cell subsets. The selective loss of MDSCs was accompanied by an increase in the antitumor activity of CD8 T and NK cells.[36] Most platinum-based compounds inhibit STAT6-regulated expression of programmed death ligand-2, thus limiting immunosuppression by both DCs and tumor cells.[74] Low-dose CTX selectively, but transiently, suppresses Tregs[43,75,76] and impairs the production of immune-suppressive cytokines, such as IL-4, IL-10 and IL-13.[41,77] A prolonged and more effective Treg inhibition was achieved by metronomic CTX regimens in patients with advanced solid tumors[6] or with metastatic breast cancer.[78] Moreover, metronomic temozolomide, an analog of dacarbazine, reduced the number and the suppressive function of circulating Tregs in rats bearing glioma, although it did not restrain tumor growth.[79]

The role of CTX on MDSCs is more controversial. Early reports have supported the concept that CTX induces the development of natural suppressor cells.[80–82] Increased circulating MDSC frequencies were observed in breast cancer patients and in B16 melanoma-bearing mice injected with CTX plus doxorubicin.[83,84] Recently, low-dose CTX treatment of mice spontaneously developing melanomas led to an accumulation of inflammatory mediators, such as GM-CSF, IL-1β, IL-5, IL-10, IFN-γ and TNF-α, in skin tumors and metastatic LNs, inducing accumulation and activation of MDSCs that abrogated CTX antitumor effects.[85] Other reports, however, show that CTX induces early myeloid effector cells that may inhibit tumor cell growth through nitric oxide (NO) release,[86] and that metronomic CTX plus gemcitabine mitigates Treg- and MDSC-mediated immunosuppression and elicits antitumor immunity *in vivo*.[87]

Among taxanes, paclitaxel specifically impairs viability and cytokine production in FOXP3$^+$ Treg cells, but not in FOXP3$^-$ CD4 effector cells.[88] Docetaxel, another antimicrotubule agent, was shown to polarize MDSCs toward an M1-like phenotype, and to selectively kill MDSCs while sparing the M1-like cells.[89] Recently, 5-FU has been shown to selectively induce MDSC apoptosis *in vitro* and *in vivo*,[49,90] resulting in enhanced IFN-γ secretion by tumor-infiltrating CD8 T-cells and T-cell-mediated antitumor responses *in vivo*.[90]

Suppressor cells adopt various means to inhibit the antitumor activity of effector lymphocytes. Some studies suggest that several enzymes, such as arginase I, indoleamine 2, 3-dioxygenase (IDO) and iNOS, as well as surface molecules, such as latency-associated peptide and CD124, are related to immune suppression and tumor progression.[68,91] Therefore, the effects of chemotherapy on these suppressive mediators also appear to be relevant for rupture of tumor-induced tolerance. A serial analysis of blood samples from advanced non-small cell lung cancer patients treated with platinum-based compounds revealed decreased iNOS, IDO and CD124 expression after chemotherapy.[92] In contrast, gemcitabine and 5-FU were recently shown to activate immune regulatory cells, which stimulated the emergence of pro-tumorigenic cytokines via inflammasome pathways, thus limiting the therapeutic efficacy of the drugs.[66]

Effects on Tumor Cells and on Tumor Microenvironment

TCD is the main goal of chemotherapy. Cytotoxic drugs kill tumor cells in different ways and modulate the host immune system accordingly, with consequences that are only now beginning to be elucidated. In addition, there is now evidence that the nature of the immune infiltrate, which often outnumbers neoplastic cells, is relevant for cancer prognosis.[93] Therefore, changing the composition of the immune infiltrate by anticancer treatments may prove beneficial for cancer elimination. Under defined circumstances, chemotherapy-induced TCD can set the stage for an effective antitumor immune response. For example, some anticancer drugs increase the expression of death receptors, including FAS, TNF receptors and TNF-related apoptosis-inducing ligand receptors.[94] Other drugs trigger apoptosis by inducing release of cytochrome *c* from mitochondria.[94] The degree of TCD correlates with clinical outcome in several tumor settings.[95,96]

Some chemotherapeutics, including anthracyclines, oxaliplatin and CTX, are unique in their capacity to induce an immunogenic type of TCD,[21,97] thereby converting dying tumor cells into adjuvanted-endogenous vaccines. The

rational base of vaccination is that tumor antigens must be captured by activated DCs, which would activate CD4 and CD8 T-cell-mediated adaptive immune responses. In an immunogenic type of TCD, antigen is provided by the dying tumor cells in the context of an immunostimulatory environment for DCs. The molecular mechanisms that distinguish immunogenic from non-immunogenic cell death have been elucidated and rely on at least three independent events: (i) early surface exposure of calreticulin (ecto-CRT) on stressed cells, (ii) subsequent secretion of ATP and (iii) release of high-mobility group box-1 (HMGB1) and HSPs by dying tumor cells.[1,98] Ecto-CRT favors the engulfment of apoptotic bodies by DCs, whereas HMGB1 and ATP modulate DC-mediated tumor antigen cross-presentation and T-cell polarization. Recently, ATP was shown to also mediate the recruitment and differentiation of myeloid DC to the tumor site following anthracycline treatment in mice.[99] Exposure of human colon carcinoma cells to a multidrug regimen, including gemcitabine, oxaliplatin, leucovorin and 5-FU induced high levels of tumor necrosis and apoptosis that activated DC cross-presentation and stimulated potent antigen-specific CTL responses *in vitro*.[100] Similarly to anthracyclines, some alkylating agents have also been shown to induce an immunogenic TCD that stimulates antigen cross-presentation by DC, and CD8 T-cell cross-priming.[21,101]

Seldom, non-apoptotic death pathways are also induced by chemotherapy with mechanisms that are now beginning to be explained. Some alkylating agents (nitrogen mustard and N-methyl-N-nitro-N-nitrosoguanidine) induce necrosis, an unregulated process rising from acute cellular stress or massive cell injury.[102] Unlike apoptosis, necrosis is intrinsically immunogenic due to the immediate release of proinflammatory mediators, such as IL-8, IL-10, TNF-α and HMGB1.[102] Paclitaxel induces mitotic catastrophe, a type of cell death that occurs as a consequence of failed mitosis.[102] Tumor cells undergoing mitotic catastrophe often have checkpoint deficiencies that result in incomplete DNA repair, replicative infidelity and chromosomal desegregation.[103] The immunogenic potential of tumor cells dying by mitotic catastrophe has not been fully clarified.

A number of antineoplastic therapies were shown to induce autophagy in human cancer cells.[104] However, whether autophagy contributes to TCD after cytotoxic therapy or represents a mechanism of resistance is still a matter of debate. During unfavorable metabolic conditions (for example, cell stress/damage by cytotoxic compounds), apoptosis-defective tumor cells can survive by invoking a protective autophagic process, that is, degradation of proteins and organelles to provide amino acids, fatty acids and nucleotides for reuse.[102,104] Some findings suggest that prolonged stimulation of autophagy may be detrimental to cancer cells and that therapies that inhibit autophagy may lead to enhanced tumor growth.[105] Other studies, however, support the use of autophagy inhibitors as potentiators of anticancer agents.[106,107] As an example, the mammalian target of rapamycin (mTOR) kinase inhibitor everolimus, which induces autophagy, prolonged the survival of patients affected by renal cell carcinoma in a phase-III clinical trial.[106] Interestingly, Michaud *et al.*[108] reported that autophagy is dispensable for chemotherapy-induced TCD, but is required for its immunogenicity.[108] These findings imply that only patients bearing autophagy-competent cancers might benefit from immunogenic chemotherapy.

Chemotherapy can also render cancer cells more susceptible to CTL and NK-cell killing. 5-FU, irinotecan and cisplatin were all shown to increase the sensitivity of the SW480 colon cancer cell line to CTLs.[109] Paclitaxel, cisplatin and doxorubicin sensitize tumor cells to CTLs by rendering them permeable to granzyme B via upregulation of mannose-6-phosphate receptors on tumor cell surface.[110] Furthermore, CTX sensitizes tumor cells to TRAIL-dependent CD8 T-cell-mediated immune attack,[111] suggesting that TRAIL-mediated tumor cell killing contributes to immunogenic TCD.[112] Platinum derivatives and dacarbazine were shown to stimulate the expression of ligands for NKG2D, an NK cell-activating receptor, resulting in augmented NK-cell cytotoxicity and IFN-γ production.[113] Numerous agents promote functional downregulation of the inhibitory NK ligand Clr-b and upregulation of stimulatory NKG2D ligand on tumor cells, thus enhancing the susceptibility of target cells to NK cell-mediated lysis.[114] Interestingly, gemcitabine has been shown to increase the expression of HLA on malignant cells,[115] and to enhance the cross-presentation of tumor antigens to CD8 T-cells.[115,116] Similarly, combined 5-FU/IFN-α treatment increased the expression of MHC class I and NKG2D ligands on murine pancreatic tumor cells.[9] In another study, low-dose chemotherapy triggered the expression of ligands for NKG2D and DNAM-1 on multiple myeloma, and promoted NK-cell degranulation against tumor.[117]

Other Off-Target, Non-Immune-Based Effects of Chemotherapy

Myelosuppression, which develops after cytotoxic chemotherapy, represents the major toxic side effect of cancer treatment, thus limiting its use. As the BM contains the most mitotically active cells in the organism, it becomes a preferential target for chemotherapy-induced cytotoxicity. However, it is now becoming clearer that not all side effects of cytotoxic chemotherapy are necessarily harmful. Genome-wide expression analysis of different tissue samples from CTX-treated tumor-bearing animals revealed the occurrence of an immunogenic apoptosis not only in tumor cells but also in BM and spleen cells, which paralleled with activation of bystander inflammatory responses.[43]

Moreover, the drug type and dosage crucially dictate the outcome of drug-induced cytotoxicity. For example, it has been reported that CTX and 5-FU are less damaging for most primitive cells than other cytotoxic drugs.[118] Furthermore, although high-dose CTX kills BM-resident DC precursors, thus hampering DC mobilization at the peripheral level,[119,120] these precursors are resistant to low-dose CTX, which instead boosts the differentiation of mature DCs *in vivo* and *ex vivo*.[21,121,122] Of note, CTX-induced mobilization and maturation of DCs from their BM precursors was mediated by endogenous type I IFN.[21,55,123]

Other non-immune targets of cytotoxic chemotherapy are the endothelial cells. Indeed, the collateral damage inflicted upon dividing endothelial cells within the tumor bed indirectly

helps tumor destruction.[124] Moreover, the majority of anticancer drugs can inhibit angiogenesis.[125] Low-dose CTX exhibits antiangiogenic activity by killing circulating endothelial progenitors[126] and decreasing NO concentrations in serum.[127] A more comprehensive and integrated understanding of the multifaceted off-target effects of cytotoxic drugs may help designing more efficacious combined treatments while avoiding ineffective or possibly antagonistic combinations.

Implications for Combination with Other Treatments

The observations reported above have several implications for planning future clinical trials combining chemotherapy with immunotherapy. First, cytotoxic agents that elicit immunogenic TCD, which converts the tumor itself into an endogenous vaccine and provides adequate DC stimulation, through release of danger signals, are ideal candidates for combination with adoptive immunotherapy strategies aimed at eliminating immune suppressor cells. For example, combining standard chemotherapy with ipilimumab, a human anti-CTLA-4 monoclonal antibody that blocks the CTLA-4 inhibitory signal on T-cells, proved extremely effective in a phase-III clinical trial on advanced melanoma patients,[128,129] as well as in a phase-II clinical trial on lung cancer patients.[130] Second, as chemotherapy increases the immune visibility of surviving or damaged tumor cells by upregulating HLA molecules and NK-related or TRAIL ligands, co-administration of immunostimulatory molecules (for example, TLR ligands, cytokines, and so on) may further facilitate immune recognition by DCs and NK, thus potentiating antitumor T-cell responses. As type I IFN enhances the cross-presentation of tumor-derived antigens by DCs[131] and synergizes with CTX when injected intratumorally,[21] these cytokines are attractive candidates to be combined with chemotherapy. Third, the selective depletion of inhibitory subsets by some anticancer drugs (for example, CTX or gemcitabine) or regimens (for example, metronomic) provides the optimal setting for combination, with active vaccination strategies aimed at expanding the already existing tumor-reactive immune responses. In this respect, several targeted compounds such as imatinib (an IDO inhibitor), sunitinib (MDSC and Treg antagonist) are also gaining applicability.[132] Fourth, some agents exert bystander effects on the host, which are crucially required for synergism with active or adoptive immunotherapy.[33] In all cases, the time-window for optimal combination must be carefully considered. Data obtained in both animal models and humans suggest that immunotherapy should immediately follow chemotherapy (1–2 days interval) to achieve the best synergism between the two treatments.[33]

Conclusions

It is now becoming evident that standard chemotherapy agents can deeply have an impact on both tumor and host immune system. Although to our knowledge no systematic analysis has been carried out to evaluate differences in the immune-based effects of conventional chemotherapeutic agents depending on cancer histology or stage, it is now clear that the existence of tumor–host interplay dictates the magnitude, quality and efficacy of most anticancer strategies. Advances in tumor immunology have now undisclosed some key mechanisms that represent the basis of therapeutic synergy or of antagonism with other treatments. The ensemble of results discussed herein contributes to pave the way towards mechanism-based, rather than empirical, rationales for combination of specific chemotherapeutic agents with selective immunotherapeutic interventions, opening novel horizons for more effective management of cancer patients.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by grants from the Italian Association for Research against Cancer (AIRC) to LB, FB and LG. We wish to thank Mrs. Rosina Bellizzi and Mr Teodoro Squatriti for secretarial and technical assistance.

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