Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis

Giovanna Schiavoni, Antonella Sistigu, Mara Valentini, Fabrizio Mattei, Paola Sestili, Francesca Spadaro, Massimo Sanchez, Silvia Lorenzi, Maria Teresa D’Urso, Filippo Belardelli, Lucia Gabriele, Enrico Proietti and Laura Bracci

Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, ITALY

Running title: CTX and IFN-I synergism in DC-driven antitumor response

Keywords: chemotherapy, type I IFN, dendritic cells, immunogenic apoptosis, homeostasis, cross-priming, cancer

Requests for reprints: Laura Bracci, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, ITALY, e-mail address:
laura.bracci@iss.it

Disclosure of potential conflict of interest: no potential conflicts of interest were disclosed

Authorship note: Giovanna Schiavoni and Antonella Sistigu contributed equally to this work
Abstract

Successful chemotherapy accounts for both tumor-related factors and host immune response. Compelling evidence suggests that some chemotherapeutic agents can induce an immunogenic type of cell death stimulating tumor-specific immunity. Here, we show that cyclophosphamide (CTX) exerts two types of actions relevant for the induction of antitumor immunity in vivo: i) effect on dendritic cell (DC) homeostasis, mediated by endogenous type I interferons (IFN-I), leading to the preferential expansion of CD8α+ DC, the main subset involved in the cross-presentation of cell-derived antigens; ii) induction of tumor cell death with clear-cut immunogenic features capable of stimulating tumor-infiltration, engulfment of tumor apoptotic material and CD8 T cell cross-priming by CD8α+ DC. Notably, the antitumor effects of CTX were efficiently amplified by IFN-I, the former providing a source of antigen and a "resetting" of the DC compartment, and the latter supplying optimal costimulation for T cell cross-priming, resulting in the induction of a strong antitumor response and tumor rejection. These results disclose new perspectives for the development of targeted and more effective chemo-immunotherapy treatments in cancer patients.
Introduction

Many clinical studies based on the combination of chemotherapy and immunotherapy have been published over the past years showing variable responses(1). Indeed, chemotherapy may be either immunostimulatory or immunosuppressive depending on the dosage and the timing of administration, and synergize with immunotherapy approaches in vivo(2-4). In addition, most chemotherapeutic agents induce tumor cell death by apoptosis, a process that has long been regarded as immunologically ‘silent’(5). However, recent evidence suggest that some anticancer drugs, such as anthracyclines, induce an immunogenic type of apoptosis that stimulates the engulfment of apoptotic bodies by dendritic cells (DC) and the activation of cytotoxic CD8 T cells, through a process known as “cross-priming”(6). Elicitation of immunogenic cell death by chemotherapeutics is characterized by a series of events that include pre-apoptotic surface translocation of calreticulin (sCRT), which serves as an “eat-me” signal for phagocytes, and the release of high-mobility group box1 protein (HMGB1) in the extracellular milieu, whose binding to TLR4 on DC triggers adaptive antitumor responses(7, 8).

Cyclophosphamide (CTX), one of the most widely used alkylating agents for the treatment of haematological and solid malignancies, has been appreciated for its immunomodulatory properties(9). Numerous mechanisms have been suggested for CTX-induced immunomodulatory effects, including the induction of a Th2/Th1 shift in cytokine production(10), the reduction of tumor-induced suppressor T cell frequencies(11), the enhancement of long-term survival and proliferation of lymphocytes(12), the induction of a variety of soluble mediators(9). Among cytokines induced by CTX, type I interferons (IFN-I) mediate many of the effects ascribed to the drug, including the expansion of memory T lymphocytes(12) and the activation of CD11b⁺ myeloid cells(13). Moreover, the efficacy of combined CTX-immune cell therapy in murine tumors was shown to be strictly dependent on endogenous IFN-I(14, 15). Recent studies suggest that CTX
immunopotentiating activity can also involve systemic mobilization of DC(16-18), although the impact of these homeostatic rearrangements on DC/tumor interaction remains elusive. One critical feature of DC for inducing efficient antitumor response is the capacity to cross-present tumor-associated antigens (Ag) and to cross-prime cytotoxic T cells, a process requiring appropriate activation stimuli(19, 20). Among signals capable of ‘licensing’ DC, IFN-I have been described to stimulate DC activation, homeostasis, migration, T cell priming and cross-priming(21-25). Indeed, IFN-I are cytokines with a long record of clinical use for the treatment of several types of malignancies due to their capacity to exert antitumor activity through multiple mechanisms(26).

Here, we analyzed the local and systemic effects of CTX in mice bearing OVA-expressing EG7 thymoma (EG7) and the synergism with IFN-I. We show that CTX-stimulated systemic DC homeostasis requires IFN-I and results in preferential expansion of CD8α+ DC. Locally, CTX induces an immunogenic tumor apoptosis, characterized by sCRT exposure and release of soluble factors, among which HMGB1, capable of activating CD8α+ DC, which efficiently take-up tumor apoptotic cells and cross-present the EG7-derived OVA both in vitro and at the tumor site. Finally, we show that CD8 T cell cross-priming by DC and CTX-induced antitumor effect in vivo can be strongly enhanced by IFN-I.
Materials and Methods

Cell lines. Rauscher virus-transformed RBL-5 lymphoma cells, originally obtained from Dr. Ion Gresser (Villejuif, Paris), and EL-4 lymphoma cells, obtained by American Type Culture Collection (ATCC, TIB-39), were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 0.1 U/ml penicillin, 0.1 mg/ml streptomycin and 0.05 mM 2-mercapto-ethanol. EG.7-OVA cells (EG7; obtained from ATCC, CRL-2113), are OVA-transfected EL4 cells and were cultured in similar medium supplemented with 0.4 mg/ml G418 (Calbiochem). OVA expression on MHC-I molecules of EG7 cells was routinely checked by flow cytometry. B16-F10 melanoma cells (obtained from ATCC; CRL-6475) were maintained in IMDM complete medium. Each cell line was routinely tested for morphology, growth curve and absence of Mycoplasma, and passaged for no more than 5 times from thawing.

Reagents and mice treatments. Mafosfamide (4-sulfoethylthio-cyclophosphamide L-lysine, Niomech –IIT GmbH) was used at 10μM. CTX (Sigma) was injected i.p. (100 mg/kg) when tumor size reached around 12mm diameter. High titre mouse IFN-I (1.5x10^6 U/mg protein) was produced as described elsewhere(27), and was either added to cell cultures (5x10^3 IU/ml) for 18h, or injected peritumorally (10^5 IU) daily for 4 days starting from day 1 post-CTX. C57BL/6, OT-1 (Charles River), and IFNAR^{-/-} mice (Dr U. Kalinke, Langen, Germany) were manipulated in accordance with the local Ethical Committee guidelines.

Bone marrow DC precursors’ analysis and culture. Bone marrow (BM) cells were collected at various times post-CTX and surface stained for detection of DC precursors (DCP) as lineage markers (Lin') MHC-II'CD11c'B220', Lin'MHC-II'CD11c'B220' and LinFlt3/CD135' then analysed by FACS. For in vitro DC differentiation, BM cells were labelled with 1 μM
carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) then cultured in medium containing 10 ng/ml rmGM-CSF (Peprotech). At various culture-times, BM-DC were surface stained for CD11c and analysed by FACS.

**Analysis of tumor-infiltrating DC.** For FACS analysis, tumor-infiltrating DC (TIDC) were detected as CD3⁻CD19⁻CD11c⁺I-A⁺ cells. For confocal laser-scanning microscopy (CLSM), frozen tumor tissue sections were fixed in acetone and stained with anti-CD11c, anti-I-A^d/I-E^d, anti-CD86, anti-MHC-I-OVAp, or Isotype. CLSM observations were performed with a Leica TCS SP2 AOBS apparatus. Signals from different fluorescent probes were taken in sequential scan settings, and co-localization was detected in yellow.

**Detection of apoptosis and immunogenicity characterization.** For apoptosis detection *in vivo*, mice were injected i.v. with green-fluorescent FLIVO™ reagent (FAM-VAD-FMK, Immunochemistry Technologies) and sacrificed 30min later. Examination of labelling in the tumor mass was performed by FACS of cell suspensions or CLSM analysis of tumor tissue sections. Immunogenic cell death of MAFO-treated EG7 cells *in vitro* was assessed by sCRT and CD31 expression by FACS and by HMGB1 release in cell culture supernatants by Western blotting. DC activation by MAFO-conditioned medium was assessed by FACS and by release of IL-6 and IL-1β. For *in vivo* assessment of immunogenic apoptosis, MAFO-treated EG7 cells were injected s.c. (30×10⁶) into one flank of C57BL/6 mice. One week later, mice were challenged with live tumor cells (5×10⁶) by s.c. injection into the opposite flank.

**Phagocytosis of apoptotic EG7 tumors and cross-priming of CD8 T cells by DC.** For uptake analysis, DC were co-cultured with apoptotic CFSE-labelled EG7 cells at a 1:4 ratio for 18h in the presence of IFN-I (5x10³ U/ml) or mock, then FACS-analysed. For proliferation assays, DC were
co-cultured with apoptotic EG7 (EG7-DC) or EL4 cells (EL4-DC), with or without IFN-I, FACS-sorted and then cultured with OT-1 CD8 T cells. $^3$H-Thymidine incorporation was measured at the third day of culture. Ag-specific IFN-γ production by CD8 T cells was assessed by ELISPOT assay following manufacturers’ instruction (Mabtech AB).

**Statistical analysis**

Levels of significance for comparison between samples were determined by the two-tailed Student’s t-test. P values minor to 0.05 were considered statistically significant.

Further details for the Materials and Methods are available online as Supplementary Data.
Results

CTX spares BM DCP and stimulates their differentiation into DC. Previous work suggests that CTX may condition DC homeostasis (16, 17), although the exact mechanisms of BM mobilization remain unclear. Here, we investigated the effect of a single injection of a lympho-depleting, non-myeloablative dose of CTX (100 mg/kg) still retaining direct anti-tumor effects (Fig. S1), on DCP in EG7 tumor-bearing mice. As shown in Fig. 1A, CTX determined a transient depletion of total BM cells, that was mostly evident at day 3 post-injection (p.i.) but not of upstream CD135^+Lin^I-A^-CD11c^- DC and downstream Lin^I-A^-CD11c^+B220^+ and B220^- DC (28, 29), which instead were significantly increased in the relative frequency (Fig. 1B and C). This effect was independent on the presence of the tumor burden (Fig. 1D). During the recovery phase (day 7-8 p.i.), when BM cell numbers raised (Fig. 1A), the rates of DCP returned similar to those found in untreated controls (Fig. 1B). These findings suggest that DCP are more resistant to low-dose CTX than other immune cell progenitors.

To investigate the proliferative and differentiation potential of DCP, we cultured CFSE-labelled BM cells with GM-CSF and analysed CFSE dilutions along with CD11c expression, as a marker for DC differentiation, at different times of culture. Consistent with the higher frequency of DCP, BM cells from day 3-CTX treated mice generated DC more rapidly with respect to controls, as determined by higher percentage of CFSE^low CD11c^+ cells appearing in BM cultures (Fig. 1E). As expected, cultures of BM isolated at day 1 or at day 9 post-CTX yielded DC with similar kinetics as compared to controls (Fig. 1E). In the periphery, CTX treatment determined a transient depletion of cDC subsets (CD8α^- and CD8α^+), but not of pDC, followed by massive de novo generation of DC resulting in the preferential expansion the CD8α^+ DC subset, confirming previous reports (Fig. S2 and Supplementary Table I) (16-18).
IFN-I critically mediate CTX-induced DC mobilization from BM. We addressed the role of IFN-I in the CTX-induced modulation of DC homeostasis. First, we analysed IFN-α and IFN-β gene expression in the BM, where mobilization of DCP originates, and found significant up-regulation of both genes in CTX-treated mice, as compared to controls, by day 3 and up to day 10 p.i. (Fig. 2A). Next, we examined DC generation potential in BM cells of IFNAR−/− animals at different times post-CTX. Remarkably, lack of IFN-I-signals strongly reduced CTX-induced DC differentiation from BM precursors in vitro, as revealed by similar CD11c+CFSElow cells retrieved in cultures from CTX-treated (day 3 p.i) and saline-treated IFNAR−/− mice at the various time-points (Fig. 2B). In contrast, BM cells from day 3 CTX-treated wild-type (WT) animals displayed significantly increased DC yield throughout all culture times, with respect to saline-treated controls (Fig. 2B). Notably, the reduced DC differentiation potential of BM cells from day 3 CTX-treated IFNAR−/− mice did not reflect a different frequency of Lin−CD135+ DCP at that time with respect to CTX-treated WT mice (Fig. 2C). Collectively, these findings indicate that IFN-I signalling is critically required for CTX-induced DC mobilization.

Induction of immunogenic tumor apoptosis by CTX. To investigate the effect of CTX on tumor cell death, we injected EG7 tumor-bearing mice with the fluorescent dye FLIVO, which binds to active caspases allowing in vivo detection of apoptosis at different times post-CTX. Remarkably, CTX largely increased the levels of apoptotic tumor cells with almost 80% of FLIVO-positivity at day 3 p.i. as opposed to control animals showing background tumor apoptosis (30-35%; Fig. 3A). Analysis of tumor sections confirmed a wide-spread distribution of FLIVO+ cells in CTX-treated mice (Fig. 3B). Notably, cell suspensions from tumor explants of CTX-treated animals failed to survive when placed in culture, while those from control mice were viable and proliferated considerably (Fig 3C).
To characterize the parameters of tumor apoptosis immunogenicity, we took advantage of the in vitro-active CTX-derivative Mafosfamide (MAFO). We found that sCRT was clearly expressed in MAFO-treated EG7 (MAFO-EG7) cells (PI gate), as compared to live tumor cells, at 4h and up to 48h post-treatment and at levels comparable to those found in UV-irradiated (UV-EG7) cells, a positive control for sCRT expression (Fig.3D). Consistently, sCRT translocation was paralleled by downregulation of the “don’t-eat-me” signal CD31 (Fig.3D). As a key parameter of cell death immunogenicity, closely related to DC activation, we measured the levels of extracellular HMGB1 in supernatant (snt) of MAFO-EG7 cells(8). Notably, both MAFO-treated and UV-irradiated EG7 cells released substantial HMGB1 (Fig. 3E). We also measured HMGB1 in snts of RBL-5 lymphoma and B16 melanoma, two cell lines displaying differential sensitivity to MAFO in vitro and to CTX in vivo (data not shown) and found both cell lines releasing HMGB1 following MAFO treatment, although B16 cells did so at lower levels with respect to EG7 and RBL-5 (Fig. 3E, F).

Lastly, to confirm the immunogenicity of MAFO-induced apoptosis in vivo, we tested MAFO-treated EG7 cells as a tumor vaccine. Strikingly, mice immunized with MAFO-EG7 cells were protected from a subsequent tumor challenge with live EG7 cells (Fig. 3G). Interestingly, vaccination with UV-EG7 cells did not protect mice from challenge, inducing only a delay in tumor progression with respect to controls (Fig. 3H). These results strongly indicate that the CTX-derivative MAFO induces an immunogenic type of apoptosis.

Phagocytosis of MAFO-‘killed’ tumor cells by CD8α⁺ DC. Since immunogenic signals of cell death promote the engulfment by phagocytes, we investigated the capacity of DC to capture MAFO-killed tumor cells. Interestingly, MAFO-EG7 cells were engulfed by CD8α⁺ DC more efficiently than UV-EG7 cells, as shown by twice higher percentages of CFSE⁺ cells (Fig. 4A). To test whether dying tumor cells released DC-activating signals, we added supernatants from UV-
EG7 or MAFO-EG7 cells to DC. Remarkably, exposure to MAFO-EG7 snt induced considerable activation of DC, as revealed by more mature phenotype of CD8α+ DC, and to a lesser extent CD8α− DC, as compared to UV-EG7 snt or medium (Fig. 4B) and by significant release of inflammatory cytokines, namely IL-1β and IL-6 (Fig. 4C). Of interest, MAFO-EG7 snt also promoted the survival of CD8α+ DC, as revealed by higher frequency of these cells after culture (Fig. 4D). No DC phenotypic changes or cytokine release were observed when MAFO was added directly to DC (data not shown), indicating that DC activation was mediated through the release of soluble factors by tumor cells after MAFO-killing. Of interest, DC activating-signals were released by MAFO-treated RBL-5, but not B16 cells, as revealed by phenotype and inflammatory cytokine release in DC upon exposure to culture snt (Fig. S3).

**Apoptotic cell uptake by DC and CD8 cross-priming are strongly enhanced by IFN-I.** CD8α+ DC are specialized for cross-presentation of dead cell-derived Ag, however appropriate activation signals are needed to license DC for cross-priming(30, 31). We asked whether IFN-I could act as such signal stimulating DC for CD8 T cell cross-priming against MAFO-EG7-derived Ag. Remarkably, in the presence of IFN-I, DC showed enhanced uptake of MAFO-EG7 cells, as indicated by 2-fold higher percentage of CD8α−CFSE+ cells, with respect to mock-treated DC (Fig. 5A). Of note, IFN-I neither affected the levels of apoptosis or those of sCRT on MAFO-treated tumor cells (data not shown). Addition of IFN-I to apoptotic cells/DC cultures induced phenotypic activation and higher levels of MHC-I-OVA peptide complexes on Ag-bearing CD8α− DC (Fig. 5B). Consistent with the enhanced phagocytosis and the more mature phenotype, IFN-treated DC were more efficient at inducing OT-1 CD8 T cell cross-priming, as revealed by higher proliferation (Fig. 5C) and by major frequencies of IFN-γ-producing cells with respect to mock-treated DC (Fig. 5D). As expected, neither proliferative response or IFN-γ-forming spots were observed when DC
loaded with MAFO-treated EL4 cells were used as stimulators, indicating the Ag-specificity of CD8 T cell response (Fig. 5C,D).

**CTX alters the tumor microenvironment promoting DC infiltration and subsequent homing to LN.** Next, we analysed whether the induction of immunogenic apoptosis and the consequent changes in tumor architecture by CTX could influence DC tumor infiltration. Notably, over an 8-fold increase in TIDC could be observed at day 7 in CTX-treated mice, with respect to untreated controls, coinciding with the peak of systemic DC expansion (Fig. 6A-B; Fig S2). A qualitative analysis of tumor sections by CLSM revealed that almost all TIDC detected in tissues from CTX-treated, but not saline-treated mice, displayed an activated phenotype, as indicated by co-localization of CD11c with CD86 and MHC-class II molecules (Fig.6C-F; Fig. S4). Of great interest, CTX-treated tumors displayed co-localization of CD11c with MHC-I-OVAp complexes, suggesting that TIDC were phagocytic and, possibly, cross-presenting EG7-derived OVA peptides on MHC-I molecules (Fig.6G-H and Fig. S4).

To test whether enhanced tumor infiltration by DC in response to CTX was driven by local alterations in chemokine balance, we analysed the intratumoral expression of selected chemokines and chemokine receptors involved in leukocyte trafficking(32). All genes analysed were significantly upregulated 3 days post-CTX, as compared to controls, supporting a scenario of a tumor microenvironment favouring DC and T cell infiltration (Fig. 6I). Moreover, the anti-angiogenic ligand/receptor pair CXCL10/CXCR3 was also upregulated in CTX-treated mice, suggesting an additional effect of this drug in inhibition of angiogenesis (Fig. 6I).

Since kinetic analysis of TIDC showed only transient tumor infiltration by these cells, which returned to the levels of controls by day 10 post-CTX (Fig. 6B), we hypothesized that after entering the tumor site, DC quickly migrate to draining LN (dLN). Thus, we injected FITC as a cell tracker intratumorally at the time of maximum tumor-infiltration (day 7 post-CTX) and investigated the
homing of TIDC to dLN. Strikingly, in CTX-treated animals, a considerable percentage of FITC<sup>+</sup>CD11c<sup>+</sup> cells migrated to dLN, but not to controlateral LN (cLN; Fig. 6L). In contrast, FITC<sup>+</sup>DC were barely detectable in dLN from saline-treated mice (Fig. 6L).

**Synergistic antitumor effect of CTX and IFN-I in vivo.** Finally, we attempted to combine systemic CTX treatment with peritumoral IFN-I administration to cure mice bearing established EG7 tumors. Notably, combined CTX/IFN treatment significantly delayed tumor development and cured 60% of mice with no tumor recurrence (Fig. 7A, B). Similar beneficial effect of combined CTX/IFN regimen was observed with mice implanted with RBL-5 tumors (Fig. 7C). As expected, mice exposed to CTX or IFN-I alone were not cured and died within 40 days (Fig. 7A-C). Importantly, mice surviving after CTX/IFN combined treatment were resistant to a subsequent tumor challenge, indicating that an immunological memory had been generated (data not shown).
Discussion

Most chemotherapeutics induce tumor cell death by apoptosis, which has been generally assumed to be immunologically silent(4). However, recent data suggest that some drugs can induce an immunogenic kind of apoptosis that stimulates antitumor immune responses contributing to tumor eradication(6, 33). Here, we have shown for the first time that CTX can induce a wide-spread tumor apoptosis with strong immunogenic features. The immunogenicity of CTX-induced cell death is demonstrated by several observations. First, the translocation of CRT on the dying cell membrane as an eat-me signal for DC paralleled by the down-regulation of the don’t-eat-me signal CD31 after treatment with the in vitro-active CTX-analogue MAFO(7). Second, the release of soluble factors, among which the alarmin protein HMGB1, promoting the activation and survival of CD8α+ DC. Third, the efficient engulfment of MAFO-killed EG7 cells by CD8α+ DC which subsequently cross-presented tumor-derived OVA peptides on MHC-I molecules in vitro and in vivo. In this regard, it is intriguing that, despite expressing similar sCRT levels, MAFO-killed EG7 were engulfed more efficiently than UV-irradiated cells by DC. This observation suggests either that additional eat-me and/or “find-me” signals may be expressed by MAFO-EG7 cells or that DC up-regulate one or more phagocytic receptors upon contact with MAFO-conditioned medium(34). Fourth, when injected into immunocompetent mice, MAFO-EG7 cells protected mice from a subsequent challenge with live tumor cells. Similarly, it was reported that tumor cells exposed to anthracyclines release strong DC-activating signals, causing immunogenic cross-presentation(8).

Although DC loaded with MAFO-EG7 cells were able to stimulate CD8 T cell cross-priming, addition of IFN-I greatly enhanced this process. In agreement with the in vitro results, IFN-I administered in vivo strongly synergized with CTX for tumor eradication. Since IFN-I treatments were performed in the local tumor microenvironment, we foresee that the beneficial effect of the cytokines may reflect an action at the DC/tumor interface. In this regard, it has been
shown that intratumoral administration of IFN-α strongly synergizes with systemic immunotherapy for induction of anti-tumor response involving enhanced DC cross-presentation(35). It is worth noting that the effectiveness of combined CTX/IFN therapy strongly correlates with susceptibility of tumor cells to CTX/MAFO-induced immunogenic cell death. In fact, RBL-5 lymphoma cells, that are sensitive to CTX-mediated immunogenic cell death, are susceptible to combined therapy in vivo. In contrast, B16 melanoma cells, which fail to undergo immunogenic apoptosis after MAFO exposure, are resistant to CTX/IFN therapy in vivo (data not shown).

Due to systemic cytotoxic effects, CTX affects lymphopoiesis and myelopoiesis, perturbing the homeostatic balance of immature myeloid cells, such as DC and myeloid-derived suppressor cells(16-18). Our results show that CTX, at non-myeloablativ doses, despite inducing transient reduction of total BM cells(16, 36), spares DCP, which, instead, increase in their relative frequency (day 3 p.i.), allowing a more rapid replenishment of the peripheral DC-compartment. Consistently, previous reports showed that promyelocytic precursor cells are less sensitive to sublethal doses of CTX, as compared to other BM progenitors, and that BM cultures from low-dose CTX-treated mice yield higher numbers of DC(37, 38). In contrast, higher doses of CTX (200 mg/kg) were shown to deplete DCP in BM of tumor-bearing mice, thus supporting the concept of a dose-dependent sensitivity of DCP to chemotherapy(17). Remarkably, CTX-mediated DCP mobilization critically required endogenous IFN-I, induced soon after CTX treatment systemically(12, 13) and in the local BM environment. Recent reports showed that IFN-I reactivate dormant HSC, promoting their proliferation and mobilization in vivo(39, 40). In addition, IFN-I can directly stimulate the turnover of DC in vivo, especially of CD8α+ DC, and promote the generation of DC from BM precursors(21, 24). Our findings support the role of IFN-I in homeostasis with crucial implications for patients undergoing myelodepleting regimens, as concomitant treatment with IFN-α could accelerate recovery of immune competence(25). Importantly, although IFN-I induction by CTX is not sufficient for tumor eradication, it is necessary for restoring immune cell pools since the
immunopotentiating activity of the drug and the effectiveness of combined CTX/immunotherapies were shown to require endogenous IFN-I to succeed(14, 15, 41). In this regard, since IFN-I was recently shown to reduce Treg cell function through stimulation of Ag-presenting cells, it is conceivable to speculate a role for endogenous IFN-I in mediating the effects of CTX on Treg cell ablation(42).

Another interesting finding reported herein is the enhanced tumor infiltration by DC following CTX-treatment. Although we cannot rule out the possibility that TIDC were recruited locally from the skin, it is intriguing that these cells appeared at the tumor site at the peak of DC frequency in lymphoid organs (day 7). The role of TIDC in tumor eradication is currently a matter of debate, although it appears that the maturation state of TIDC may crucially dictate the outcome of effector CTL responses and a positive correlation of mature TIDC with longer survival of tumor patients has been reported in clinical studies(43-45). Remarkably, in tumor tissues from CTX-treated, but not saline-treated, animals almost all TIDC displayed a mature phenotype, revealed by CD86 and MHC-II expression, and expressed MHC-I-OVAp complexes. Of note, the presence of CD11c+ DC co-expressing MHC-I-OVAp is indicative not only of active phagocytosis of dying tumor cells by TIDC, but may also suggest cross-presentation of EG7-derived OVA. The appearance of TIDC in CTX-treated mice correlated with an intratumoral chemokines/chemokine receptors milieu supporting leukocyte recruitment and trafficking, as revealed by early intratumoral upregulation of CXCR3 and CCR5, as well as of CXCL12, CCL19, CCL20 and CXCL10(32, 46, 47). Interestingly, it has been reported that the interaction between CXCR3 and its ligands and the progressive increase in CXCL10 intratumoral expression critically inhibit angiogenesis, thus suggesting a possible role for CTX in this phenomenon(32, 46, 48).

After the peak of tumor infiltration considerable numbers of DC migrated to tumor-dLN in CTX-treated mice (day 10 p.i.). Ag-bearing DC migrating from peripheral tissues to dLN can either directly present the carried Ag to naïve T cells or hand over the antigenic cargo to LN-resident
DC(49). It has been proposed that migratory DC, rather than CD8α+ DC, retain more immunogenic features thus enhancing immune responses in naïve CTX-treated mice(18). However, our data on Ag cross-presentation by CD8α+ DC and CD8 T cell cross-priming argue against the assumption that these cells may be tolerogenic, at least in a setting where tumor-derived antigenic material and immunogenic signals are made available for DC due to CTX cytotoxic activity. Thus, we propose that upon CTX-induced tumor death, activated DC leave the tumor microenvironment and migrate to dLN, where they either directly present or transfer tumor Ag to resident CD8α+ DC, previously expanded by CTX, to initiate antitumor responses. In this scenario, co-administration of IFN-I in the local intratumoral milieu functions as a powerful signal that license DC for efficient cross-priming.

Altogether, our data indicate that CTX, on one hand, induces an immunogenic apoptosis within the tumor mass that acts as priming event for the induction of antitumor immunity through the release of large amounts of antigenic material and soluble factors recruiting and activating DC into the tumor bed, and, on the other hand, resets the host immune system creating an excellent stage for homeostatic expansion of DC pools. Due to the powerful capability to promote DC-mediated CD8 T cell responses and to exert synergistic therapeutic antitumor effect in vivo, IFN-I represent promising candidates for combination therapies with CTX for the development of more effective immunotherapy protocols for cancer patients.
Acknowledgements

We are grateful to Dr. Federica Moschella and Dr. Iole Macchia for fruitful scientific discussion and critical reading of manuscript. We are also grateful to Dr. Elena Toschi for support with Western blotting, and to Sonia Maccari, Irene Canini and Anna Maria Pacca for excellent technical assistance. This work was supported by grants from Italian Ministry of University and Research (MIUR, Prot.n° RBIP063Z85), from ACC 2006 - National Program 2- (ACC2/WP5.4) and from Italian Society for Cancer Research (AIRC).
References


Figure legends

Figure 1. Effect of CTX injection on BM mobilization and DC homeostasis. EG7 tumor-bearing mice were injected i.p. with CTX or saline. At the indicated time-points BM were extracted. A, total BM cell counts in each individual mouse (mean±SD). B, relative frequency of B220⁺ and B220⁻ DCP in whole BM. C, CD135⁺ DCP at day 3 p.i. in tumor-bearing and tumor-free mice. D, B220⁺ and B220⁻ DCP in tumor-bearing and tumor-free mice. Data are representative of four independent experiments. E, GM-CSF cultures of CFSE-labeled BM cells from tumor-bearing mice at day 1, 3 and 9 p.i. Data show mean percentages of CD11c⁺CFSE<sub>low</sub> cells in triplicate cultures±SD at the indicated times. One representative experiment out of three.

Figure 2. Role of IFN-I in CTX-induced DC mobilization. A, qRT-PCR of BM at various times post-CTX. Data represent the relative amount of IFN-α and IFN-β mRNA normalized to β-actin (mean±SD). One representative experiment out of three. B, GM-CSF cultures of CFSE-labeled BM cells from IFNAR<sup>-/-</sup> and WT mice at day 3 p.i. Data show mean percentages of CD11c⁺CFSE<sub>low</sub> cells in triplicates±SD at the indicated culture times. *P<0.05, **P<0.01. C, DCP frequency in BM from IFNAR<sup>-/-</sup> and WT mice at various times p.i. Zero time represents saline-treated mice. Bars depict mean frequencies of Lin⁻CD135⁺ DCP in each individual mouse out of three±SD.

Figure 3. Induction of immunogenic apoptosis by CTX. Tumor-bearing mice were treated with FLIVO at the indicated times p.i. A, FACS analysis of FLIVO staining in tumor cell explants. B, CLSM of tumor sections. Bars correspond to 100µm. C, cell counts of <i>ex vivo</i>-cultured tumor explants from mice at day 3 p.i. One out three experiments is shown. D, sCRT and CD31 expression on live, MAFO-EG7 or UV-EG7 cells (PI⁻ gate). One experiment out of four is shown. E, Western blotting of HMGB1 protein expression in snt or whole cell lysates of live, UV-irradiated...
EG7 or MAFO-treated EG7, RBL-5 and B16 cells. Supernatants were normalised to cell numbers.

F, densitometry of HMGB1 expression. Data represented fold-change ratios in MAFO-treated vs.
live cell sn. G-H, growth of EG7 tumors in mice vaccinated with MAFO-treated or UV-irradiated
EG7 cells. Mean tumor diameter±SD of three mice per group. One representative experiment out of
three is shown.

Figure 4. MAFO-EG7 cell uptake by DC. A, naïve DC were co-cultured with CFSE-labeled
MAFO-EG7 or UV-EG7 cells. Uptake by CD8α⁺ DC was measured 18h later by FACS as CFSE⁺.
B, phenotype of DC subsets after 18h-culture with snTs from MAFO-EG7 (gray-opened), UV-EG7
cells (black-opened) or medium (gray-filled). C, cytokine release by DC. Data show mean±SD of
triplicate wells. D, percentage of CD8α⁺ DC. Data represent one representative experiment out of
three.

Figure 5. Effect of IFN-I on cross-presentation of EG7-derived OVA by DC. A, uptake by
CD8α⁺ DC of CFSE⁺ MAFO-EG7 cells after 18h-culture with IFN-I or mock. B, phenotype of
IFN-treated or mock-treated CD8α⁺ CFSE⁺-DC. One experiment out of three is shown. C,
proliferative response of OT-1 CD8 T cells to DC loaded with MAFO-EG7 cells plus IFN-I or
mock, with MAFO-EL4 plus IFN-I, or with OVA protein. Each point represents the mean cpm± SD
of triplicate cultures. D, OVA-specific IFNγ forming spots of OT-1 CD8 T cells after 48h-culture
with mock-DC/EG7, IFN-DC/EG7, or IFN-DC/EL4. One representative experiment out of three is
shown.

Figure 6. Tumor infiltration and LN homing of DC after CTX. A, CD11c⁺I-A⁺ TIDC at day 7
p.i. (CD3⁻CD19⁻ gate) in tumor bulk. B, kinetic analysis of TIDC in tumor explants at various times
p.i. Histograms represent mean frequencies in each individual mouse out of three±SD. **P<0.01.
One experiment out of four. C-H, analysis of TIDC in tumor sections by CLSM. Expression of CD86 (C-D), MHC-II (E-F) and MHC-I/OVAp complexes (G-H) by CD11c⁺ DC is shown by co-localization (yellow). Inserts represent high magnification portions of the fields displayed. Bars correspond to 50μm. One representative experiment out of three is presented. I, qRT-PCR analysis of chemokine/chemokine receptors in tumor bulk at different times p.i. Plots represent mRNA relative amount normalized to β-actin run in triplicate of each individual mouse out of three±SD. *P<0.05. One representative experiment out of two. L, mice were inoculated intratumorally with FITC at day 7 p.i. Density plots show the frequency of FITC⁺ DC dLN and cLN 3 days later. This experiment was repeated twice.

Figure 7. Antitumor effect of combined CTX/IFN-I treatments. Mice bearing implanted tumors were injected i.p. with CTX followed by 4 peritumoral injections of IFN-I. A, EG7 tumor growth expressed as mean diameter±SE (5 mice/group). Number of surviving mice is indicated in brackets. B, mortality over time. One representative experiment out of three. C, RBL-5 tumor growth expressed as mean diameter±SE. One experiment out of two.
Figure 1

A. Cell number (x10^6) vs. Days post injection for Saline and CTX groups.

B. % cells/total BM vs. Days post injection for B220^+ and B220^- DCP.

C. Flow cytometry images of tumor bearing and tumor free days 3, Saline and CTX.

D. % cells/total BM vs. Days post injection for B220^+ DCP, days 3 and 8, with different treatment groups.

E. % CD11c^+ cells vs. Days of culture for Saline and CTX, days 1, 3, and 9.
Figure 2

A

Day 10 Day 7 Day 3
Saline

IFN-α

0 - 0.01
1 - 0.05
2 - 0.1
3 - 0.5
4 - 1.0

Relative amount of mRNA normalized to actin

B

Day 3 p.i.

IFNAR/-

WT

Saline

% CD135+ cells

C

Days post CTX Injection

% Lin-CD135+ cells

0 - 0.5
1 - 1.0
2 - 1.5
3 - 2.0
4 - 2.5

Relative amount of mRNA normalized to actin

0 - 0.01
1 - 0.05
2 - 0.1
3 - 0.5
4 - 1.0

Days of culture
Figure 3

A. % FLVO positive cells.

B. Saline vs. CTX (48h).

C. Cell count (cells/ml x 10^6).

D. UV irradiation vs. Mafosfamide.

E. HMGB1 densitometry.

F. Mean tumor diameter (mm±SD).

G. Mean tumor diameter (mm±SD).

H. Mean tumor diameter (mm±SD).
Figure 4

A

UV-EG7

MAFO-EG7

6.6%
MFI=717

12.1%
MFI=828

CD8α

CFSE

B

CD40

CD86

MHC-I

CD8α⁺

DC

CD8α⁻

DC

Fluorescence intensity

Events

C

IL-1β

IL-6

pg/ml

0

10

20

30

0

10

20

30

DC alone
DC+UV-EG7
DC+MAFO-EG7

DC alone
DC+UV-EG7
DC+MAFO-EG7

D

Medium
UV-EG7 snt
MAFO-EG7 snt

CD8α

CD11c

15.1
72.2
29

17.5
70
59.3

0

40

80

120

160

200

DC alone
DC+UV-EG7
DC+MAFO-EG7
Figure 5

A

Mock-treated DC

IFN-treated DC

CD8α CFSE+ gate

B

CD86

CD80

CD40

MHC-I

MHC-I-OVA

Mock-treated DC

IFN-treated DC

C

\( ^{3}\text{H}-\text{TdR} \) incorporation (cpm x 10^3)

\( \text{DC:T ratio} \)

D

IFN-γ ELISPOT

Mock-DC/EG7

IFN-DC/EG7

IFN-DC/EL4
Figure 7

A) Mean tumor diameter (mm ± SE) for RBL-5 and EG7 tumors.

B) Percentage of surviving mice treated with different combinations of CTX, IFN, and saline.

C) Days after tumor implant for RBL-5 and EG7 tumors.
Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis

Giovanna Schiavoni, Antonella Sistigu, Mara Valentini, et al.

Cancer Res  Published OnlineFirst December 13, 2010.