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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

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 $CD8\alpha^+$ DC, the main subset involved in the cross-presentation of cell-derived antigens; and (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 $CD8\alpha^+$ 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 chemoimmunotherapy treatments of cancer patients. *Cancer Res; 71(3); 768–78. ©2010 AACR.*

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 may 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 preapoptotic 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 hematologic 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), and 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,

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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 a preferential expansion of $CD8\alpha^+$ DC. Locally, CTX induces an immunogenic tumor apoptosis, characterized by sCRT exposure and release of soluble factors, among which HMGB1, capable of activating $CD8\alpha^+$ DC, efficiently takes 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 (Centre de Recherches Biomédicales des Cordeliers, Paris, France), 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 fetal calf serum, 2 mmol/L L-glutamine, 0.1 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.05 mmol/L 2-mercaptoethanol. 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 Iscove's modified Dulbecco's medium 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 [(MAFO) 4-sulfoethylthio-cyclophosphamide L-lysine; Niomech–IIT GmbH] was used at 10 μmol/L. CTX (Sigma) was injected i.p. 100 mg/kg when tumor size reached around 12-mm diameter. High-titer mouse IFN-I (1.5×10^6 U/mg protein) was produced as described elsewhere (27) and was either added to cell cultures (5×10^3 IU/mL) for 18 hours or injected peritumorally (10^5 IU) daily for 4 days starting from day 1 post–CTX treatment. C57BL/6, OT-1 (Charles River), and $IFNAR^{-/-}$ mice (Dr U. Kalinke, Paul Enrich Institute, Langen, Germany) were manipulated in accordance with the local Ethical Committee guidelines.

Bone marrow DC precursor analysis and culture

Bone marrow (BM) cells were collected at various times post-CTX treatment and surface stained for detection of DC precursors (DCP) as lineage markers $(Lin)^-$ $MHC\text{-}II^-CD11c^+B220^+$, $Lin^-MHC\text{-}II^-CD11c^+B220^-$, and $Lin^-Flt3/CD135^+$ and then analyzed by FACS. For *in vitro* DC differentiation, BM cells were labeled with 1 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and then cultured in medium containing 10 ng/mL rmGM-CSF (Peprotech). At various culture times, BM DC were surface stained for CD11c and analyzed by FACS.

Analysis of tumor-infiltrating DCs

For FACS, tumor-infiltrating DC (TIDC) were detected as $CD3^-CD19^-CD11c^+I\text{-}A^+$ cells. For confocal laser-scanning microscopy (CLSM), frozen tumor tissue sections were fixed in acetone and stained with anti-CD11c, anti-$I\text{-}A^d$/$I\text{-}E^d$, anti-CD86, anti-MHC-I-OVAp, or Isotype. CLSM observations were done with a Leica TCS SP2 AOBS apparatus. Signals from different fluorescent probes were taken in sequential scan settings, and colocalization 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 30 minutes later. Examination of labeling in the tumor mass was done 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 (snt) 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. (30×10^6) into 1 flank of C57BL/6 mice. One week later, mice were challenged with live tumor cells (5×10^6) by subcutaneous injection into the opposite flank.

Phagocytosis of apoptotic EG7 tumors and cross-priming of CD8 T cells by DCs

For uptake analysis, DC were cocultured with apoptotic CFSE-labeled EG7 cells at a 1:4 ratio for 18 hours in the presence of IFN-I (5×10^3 U/mL) or mock and then analyzed by FACS. For proliferation assays, DC were cocultured 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 2-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Further details of the Materials and Methods section 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

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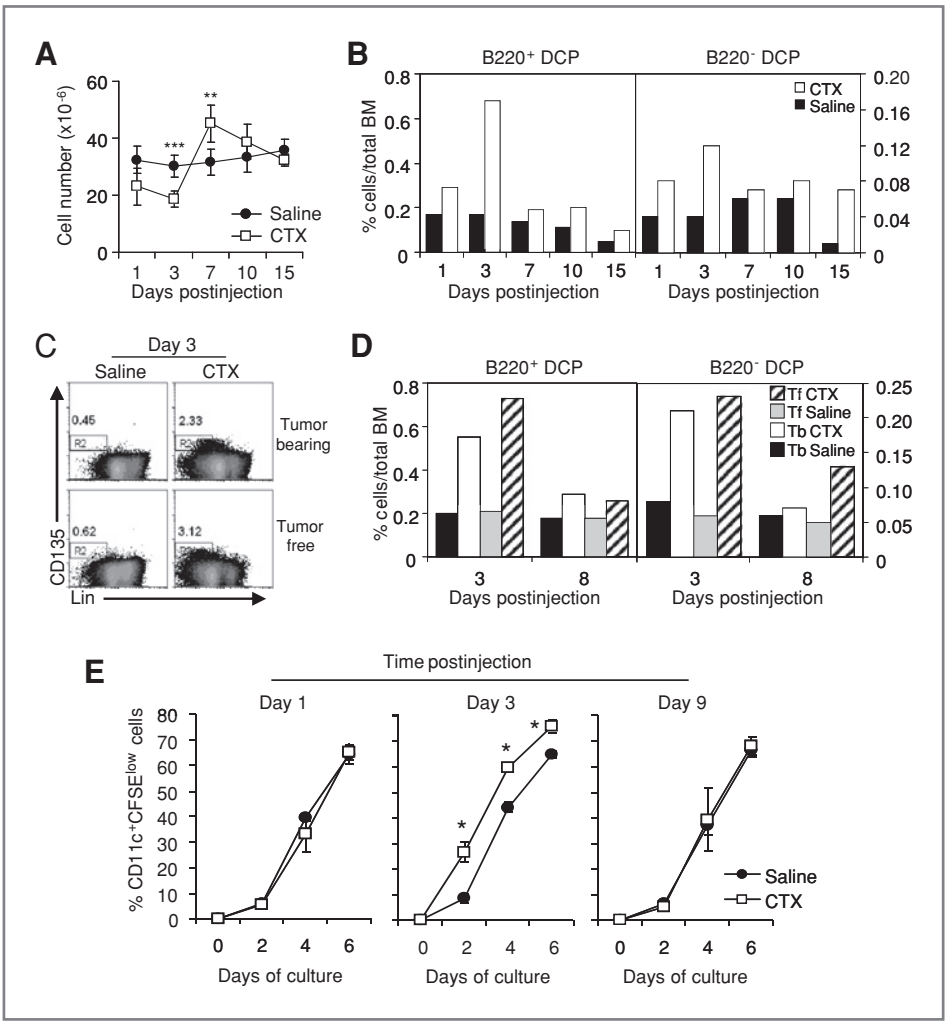

Figure 1. Effect of CTX injection on BM mobilization and DC homeostasis. EG7 tumor-bearing mice were injected i.p. with CTX or saline. At the indicated time points, BM was extracted. A, total BM cell counts in each individual mouse (mean ± SD). B, relative frequency of $B220^+$ and $B220^-$ DCPs in whole BM. C, $CD135^+$ DCPs at day 3 p.i. in tumor-bearing and tumor-free mice. D, $B220^+$ and $B220^-$ DCPs in tumor-bearing and tumor-free mice. Data are representative of 4 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 ± SD of $CD11c^+CFSE^{low}$ cells in triplicate cultures at the indicated times. One of 3 representative experiments is shown. Tb, tumor bearing; Tf, tumor free. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mobilization remain unclear. Here, we investigated the effect of a single injection of a lymphodepleting, nonmyeloablative dose of CTX (100 mg/kg), still retaining direct antitumor effects (Supplementary Fig. S1), on DCP in EG7 tumor-bearing mice. As shown in Figure 1A, CTX determined a transient depletion of total BM cells that was mostly evident at day 3 postinjection (p.i.) but not of upstream $CD135^+Lin^-I\text{-}A^-CD11c^-$ DCP and downstream $Lin^-I\text{-}A^-CD11c^+B220^+$ and $B220^-$ DCP (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 increased (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-labeled BM cells with GM-CSF (granulocyte macrophage colony stimulating factor) and analyzed 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 DCs 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 treatment yielded DC with similar kinetics as compared with controls (Fig. 1E). In the periphery, CTX treatment determined a transient depletion of conventional DC subsets ($CD8\alpha^+$ and $CD8\alpha^-$), but not of plasmacytoid DC, followed by massive *de novo* generation of DC resulting in the preferential expansion of the $CD8\alpha^+$ DC subset, confirming previous reports (Supplementary Fig. S2 and Supplementary Table I; refs. 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 analyzed IFN-α and IFN-β gene expression in the BM, where mobilization of DCP originates, and found significant upregulation of both genes in CTX-treated mice, as compared with controls, by day 3 and up

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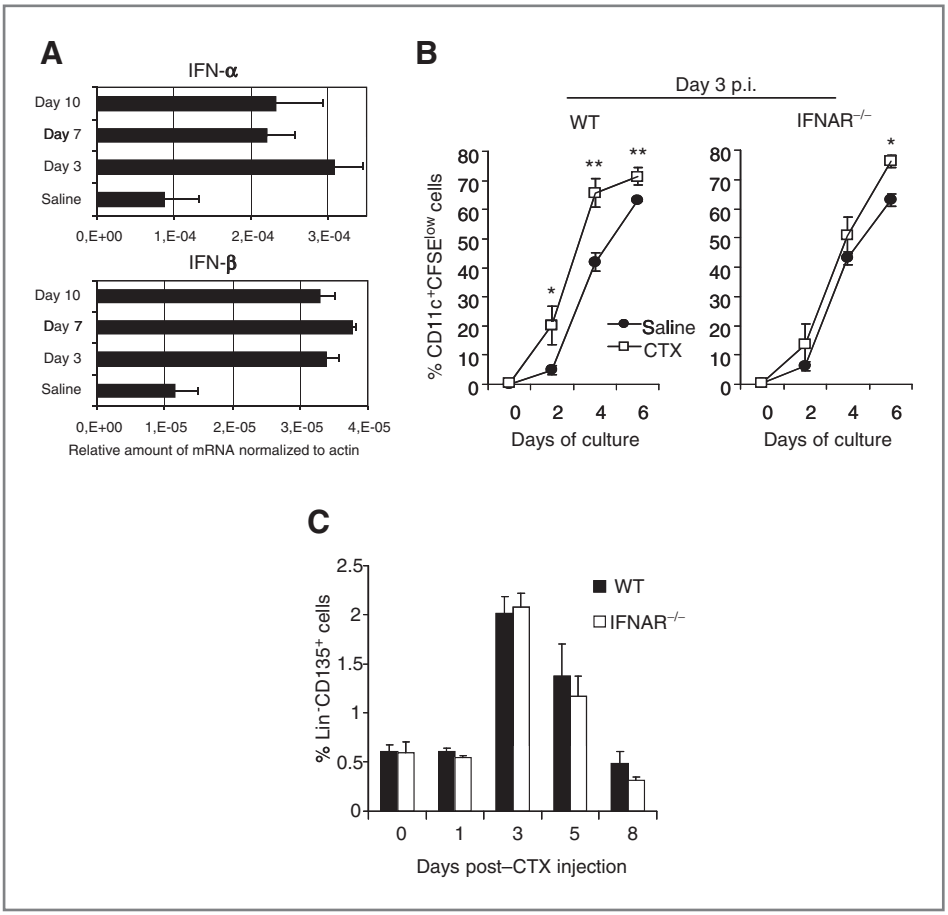

Figure 2. Role of IFN-I in CTX-induced DC mobilization. A, quantitative reverse transcriptase PCR (qRT-PCR) of BM at various time points post–CTX treatment. Data represent the relative amount of IFN-α and IFN-β mRNA normalized to β-actin (mean ± SD). One of 3 representative experiments is shown. B, GM-CSF cultures of CFSE-labeled BM cells from IFNAR$^{-/-}$ and WT mice at day 3 p.i. Data show mean percentages ± SD of CD11c^{+}CFSE^{low} cells in triplicates at the indicated culture times. *, $P < 0.05$; **, $P < 0.01$. C, DCP frequency in BM from IFNAR$^{-/-}$ and WT mice at various times p.i. Zero time represents saline-treated mice. Bars depict mean frequencies of Lin^{-}CD135^{+} DCP in 1 of 3 individual mice ± SD.

to day 10 p.i. (Fig. 2A). Next, we examined DC generation potential in BM cells of IFNAR$^{-/-}$ animals at different times post—CTX treatment. Remarkably, lack of IFN-I signals strongly reduced CTX-induced DC differentiation from BM precursors *in vitro*, as revealed by similar CD11c^{+}CFSE^{low} 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 signaling 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 treatment. 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). The analysis of tumor sections confirmed a widespread 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, whereas 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 MAFO. We found that sCRT was clearly expressed in MAFO-treated EG7 (MAFO-EG7) cells (PI^{-} gate), as compared with live tumor cells, at 4 hour and up to 48 hour post-treatment and at levels comparable with 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 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 than EG7 and RBL-5 (Fig. 3E and F).

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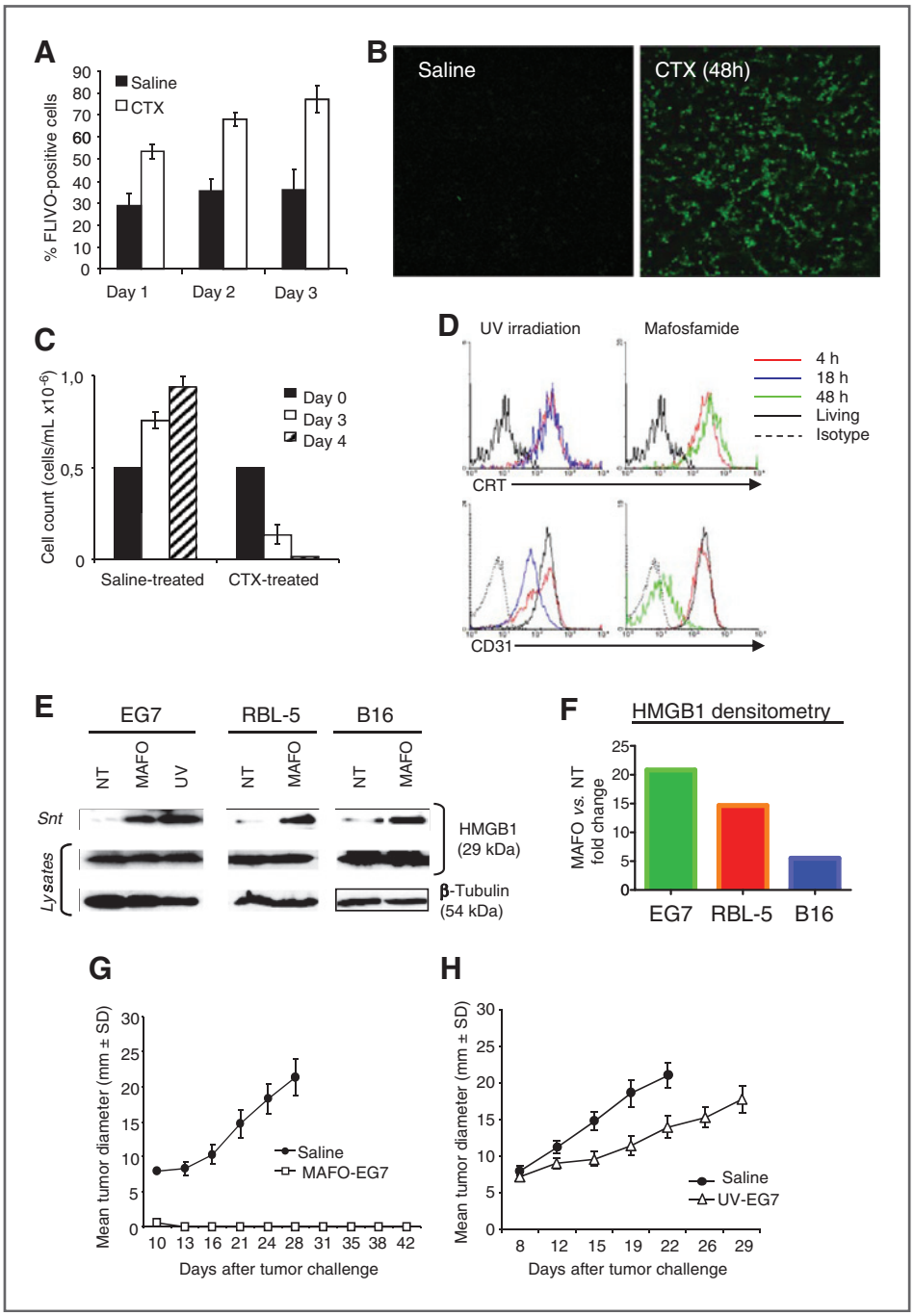

Figure 3. Induction of immunogenic apoptosis by CTX. Tumor-bearing mice were treated with FLIVO at the indicated times p.i. A, FACS of FLIVO staining in tumor cell explants. B, CLSM of tumor sections. Bars, 100 μm. C, cell counts of *ex vivo* cultured tumor explants from mice at day 3 p.i. One of 3 experiments is shown. D, sCRT and CD31 expression on live, MAFO-EG7, or UV-EG7 cells (PI^- gate). One of 4 experiments 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 normalized to cell numbers. F, densitometry of HMGB1 expression. Data represent fold-change ratios in MAFO-treated versus live cell snts. G and H, growth of EG7 tumors in mice vaccinated with MAFO-treated or UV-irradiated EG7 cells. Mean tumor diameter ± SD of 3 mice per group. One of 3 representative experiments is shown.

Finally, 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

Because 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 snts from UV-EG7 or MAFO-EG7

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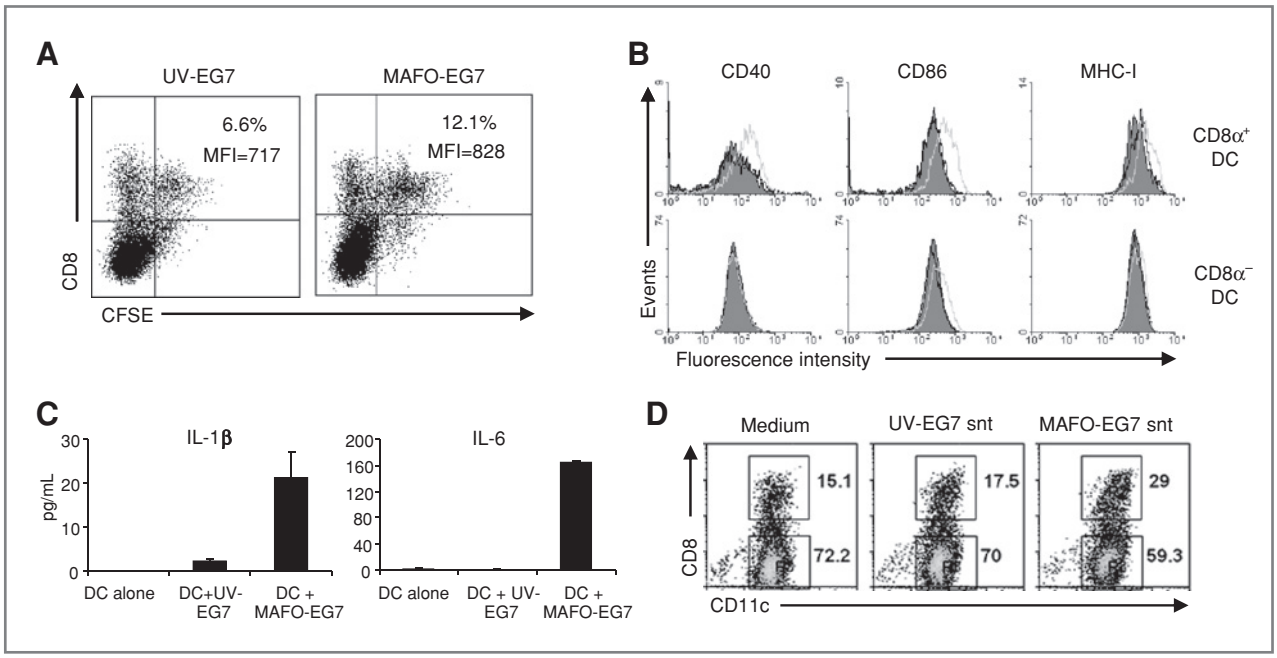

Figure 4. MAFO-EG7 cell uptake by DC. A, naive DC were cocultured with CFSE-labeled MAFO-EG7 or UV-EG7 cells. Uptake by CD8α$^+$ DC was measured 18 hours later by FACS as CFSE$^+$. B, phenotype of DC subsets after 18-hour culture with snts from MAFO-EG7 (gray opened), UV-EG7 cells (black opened), or medium (gray filled). C, cytokine release by DC. Data show mean ± SD of triplicate wells. D, percentage of CD8α$^+$ DC. Data represent 1 of 3 representative experiments.

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 with UV-EG7 snt or medium (Fig. 4B) and by significant release of inflammatory cytokines, namely, IL-1α 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 was 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 on exposure to culture snt (Supplementary Fig. S3).

Apoptotic cell uptake by DC and CD8 cross-priming are strongly enhanced by IFN-I

CD8α$^+$DCs 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 than mock-treated DC (Fig. 5A). Of note, IFN-I neither affected the levels of apoptosis nor affected 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 nor 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 and D).

CTX alters the tumor microenvironment promoting DC infiltration and subsequent homing to lymph node

Next, we analyzed whether the induction of immunogenic apoptosis and the consequent changes in tumor architecture by CTX could influence DC tumor infiltration. Notably, a more than 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 and B; Supplementary 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 colocalization of CD11c with CD86 and MHC-II molecules (Fig. 6C–F; Supplementary Fig. S4). Of great interest, CTX-treated tumors displayed colocalization of CD11c with MHC-I-OVAp complexes, suggesting that TIDCs were phagocytic and, possibly, cross-presenting EG7-derived OVA peptides on MHC-I molecules (Fig. 6G and H; Supplementary Fig. S4).

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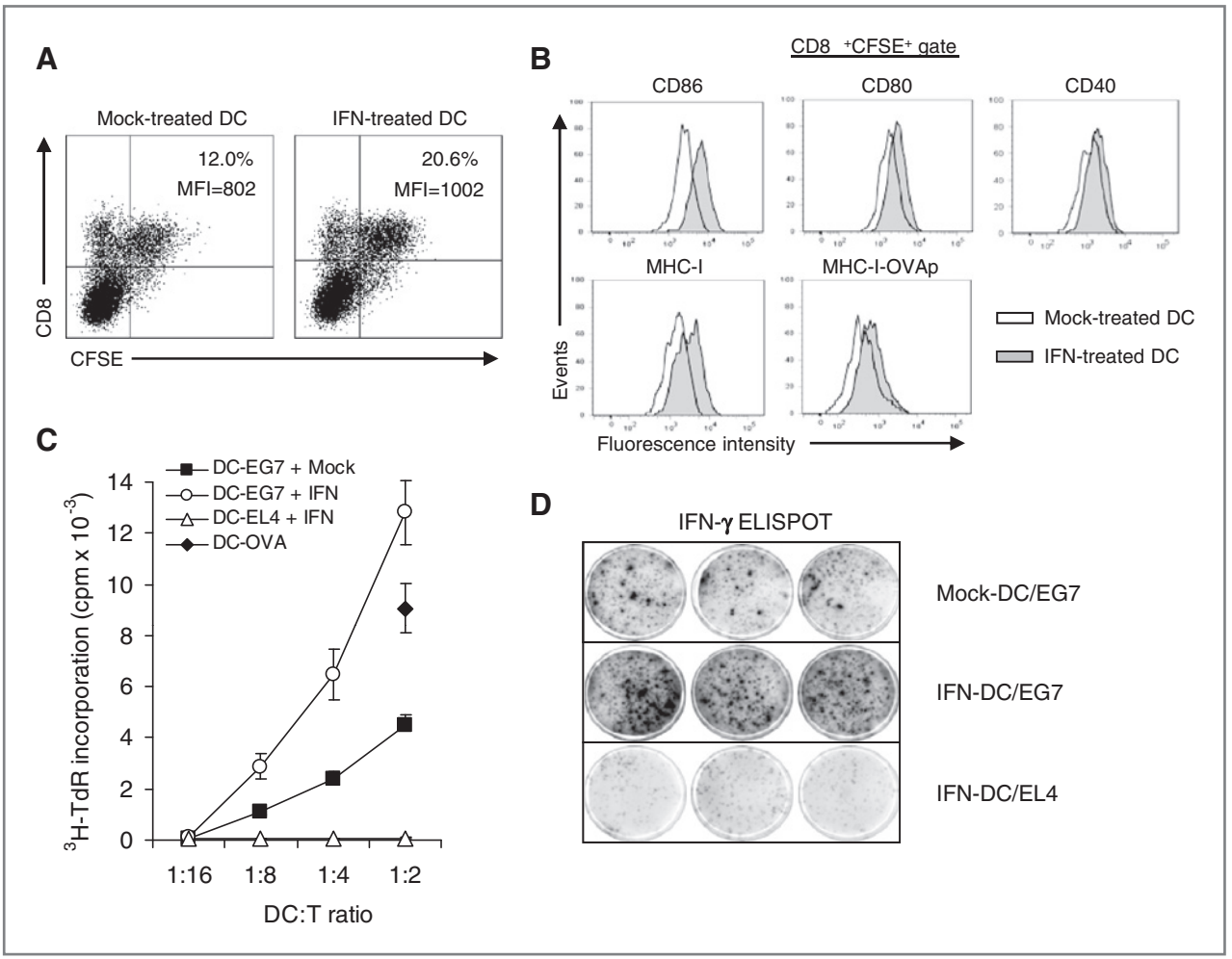

Figure 5. Effect of IFN-I on cross-presentation of EG7-derived OVA by DC. A, uptake by CD8α$^+$ DC of CFSE$^+$ MAFO-EG7 cells after 18-hour culture with IFN-I or mock. B, phenotype of IFN-treated or mock-treated CD8α$^+$ CFSE$^+$ DC. One of 3 experiments is shown. C, proliferative response of OT-1 CD8 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 counts per million (cpm) ± SD of triplicate cultures. D, OVA-specific IFN-γ–forming spots of OT-1 CD8 T cells after 48-hour culture with mock-DC/EG7, IFN-DC/EG7, or IFN-DC/EL4. One of 3 representative experiments is shown.

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

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

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 and 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 to C). Importantly, mice

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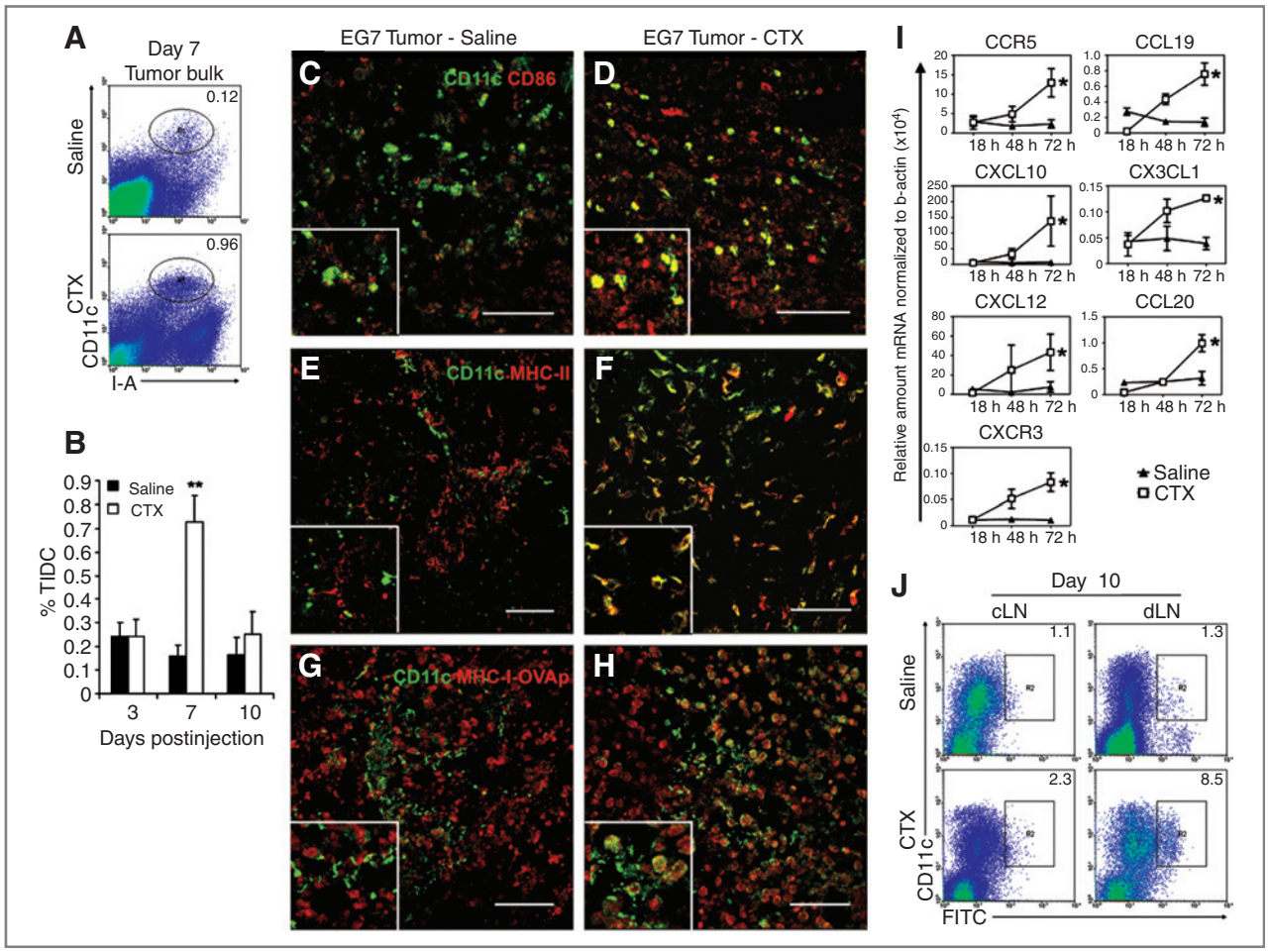

Figure 6. Tumor infiltration and LN homing of DC after CTX. A, $CD11c^+I\text{-}A^+$ TIDC at day 7 p.i. ($CD3^-CD19^-$ gate) in tumor bulk. B, kinetic analysis of TIDC in tumor explants at various times p.i. Histograms represent mean frequencies ± SD of 1 of 3 individual mice. **, $P < 0.01$. One of 4 experiments is shown. C to H, analysis of TIDC in tumor sections by CLSM. Expression of CD86 (C and D), MHC-II (E and F), and MHC-I/OVAp complexes (G and H) by $CD11c^+$ DC is shown by colocalization (yellow). Inserts represent high magnification portions of the fields displayed. Bars, 50 μm. One of 3 representative experiments 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 1 of 3 individual mice ± SD. *, $P < 0.05$. One of 2 representative experiments is shown. J, 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.

surviving after CTX/IFN combined treatment were resistant to a subsequent tumor challenge, indicating that an immunologic 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 widespread tumor apoptosis with strong immunogenic features. The immunogenicity of CTX-induced cell death is shown by several observations. First, the translocation of CRT on the dying cell membrane as an "eat me" signal for DC paralleled by the downregulation 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 $CD8\alpha^+$ DC. Third, the efficient engulfment of MAFO-killed EG7 cells by $CD8\alpha^+$ 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 upregulate one or more phagocytic receptors on 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).

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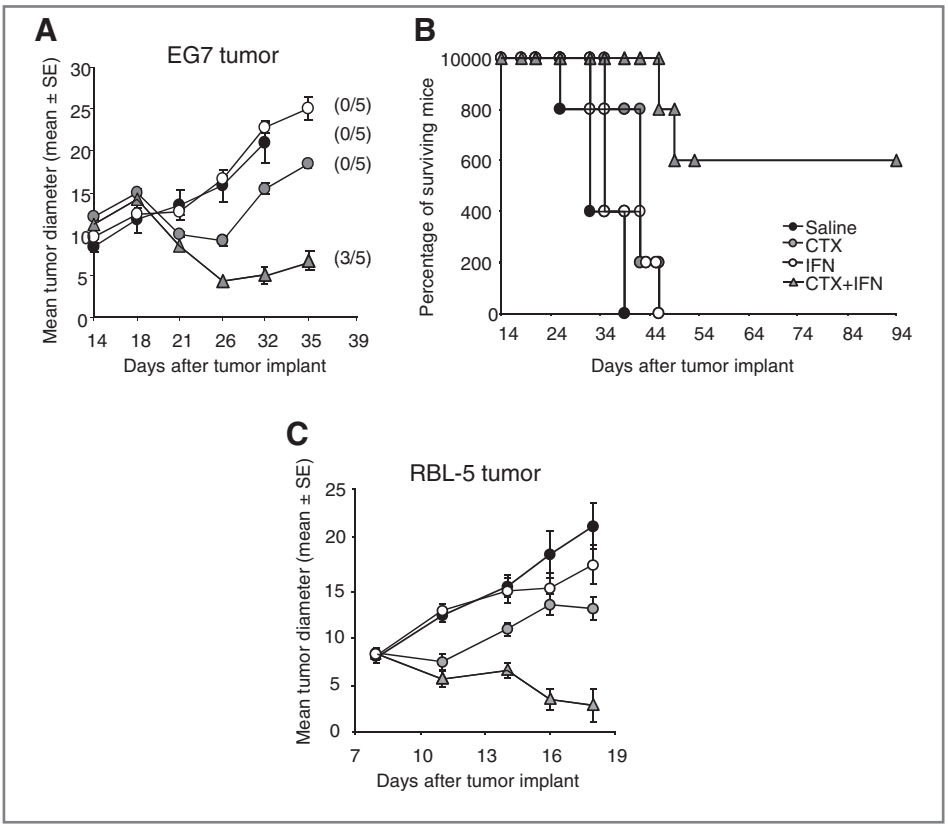

Figure 7. Antitumor effect of combined CTX/IFN-I treatments. Mice bearing implanted tumors were injected i.p. with CTX, followed by 4 peritumoral injections of IFN-I. A, EG7 tumor growth expressed as mean diameter ± SE (5 mice/group). Number of surviving mice is indicated in brackets. B, mortality over time. One of 3 representative experiments is shown. C, RBL-5 tumor growth expressed as mean diameter ± SE. One of 2 experiments is shown.

Although DC loaded with MAFO-EG7 cells could stimulate CD8 T-cell cross-priming, the 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. Because IFN-I treatments were done 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 the induction of antitumor 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, which 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).

Because of 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-myeloablative 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 than 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 hematopoietic stem cells, promoting their proliferation and mobilization *in vivo* (39, 40). In addition, IFN-I can directly stimulate the turnover of DC *in vivo*, especially of $CD8\alpha^+$ 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 because 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, because IFN-I was recently shown to reduce regulatory T cell (Treg) 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 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 seems 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 coexpressing 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, and also 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 naive T cells or hand over the antigenic cargo to LN-resident DC (49). It has been proposed that migratory DC, rather than $CD8\alpha^+$ DC, retain more immunogenic features, thus enhancing immune responses in naive CTX-treated mice (18). However, our data on Ag cross-presentation by $CD8\alpha^+$ 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 on 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 $CD8\alpha^+$ DC, previously expanded by CTX, to initiate antitumor responses. In this scenario, coadministration of IFN-I in the local intratumoral milieu functions as a powerful signal that licenses 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. Because of 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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