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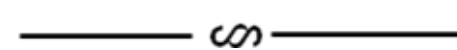

Risposte immuni e infiammatorie indotte dalla chemioterapia

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1. INTRODUCTION

A daunting diversity of distinct molecular etiologies gives rise to one class of life-threatening diseases — cancer [1, 2] — which affects half of the inhabitants of developed countries during their lifetime and kills one-third of them.

Cancer is widely considered a cell-autonomous genetic disease that results from epigenetic and genetic reprogramming in oncogenes, tumor-suppressor genes and genome-stability genes, all these being essential players in both oncogenesis and tumor progression.

As defined primarily by Hanahan and Weinberg, the tumorigenic process stems from six hallmark criteria i.e., growth signal self-sufficiency, resistance to growth-inhibitory signals, resistance to apoptosis, limitless growth potential, sustained angiogenesis, and metastasizing potential [1]. As an ancillary proposition tumors are more than insular masses of proliferating cancer cells. Instead, they are complex tissues of multiple distinct cell types (cancer cells, stromal cells, immune cells and the extracellular matrix) that participate, in a silent movie, in heterotypic interactions with one another. Such ensemble of cells is a main battleground during the neoplastic process, fostering proliferation, survival and migration of tumor cells. Indeed, for the development of full-blown neoplasia, cancer cells must overcome intrinsic (cell autonomous) and extrinsic (immune mediated) barriers to oncogenesis [3]. Only when tumor cells overreach immune control they can progress. As recently proposed by Schreiber and colleagues [4, 5], avoidance of immunosurveillance might be the seventh hallmark of cancer.

Comprehensive information on the tumor and the immune status of an individual could be expected to provide a precise picture of the ongoing evolution of the tumor (and therefore a useful tool for prognostic extrapolation), as well as to yield invaluable information about which strategy (surgery, chemotherapy, radiotherapy and/or immunotherapy) will result in an optimal therapeutic outcome.

1.1 CANCER DESPITE IMMUNOSURVEILLANCE: IMMUNOEDITING AND IMMUNOSUBVERSION

Comments made decades ago by Burnet and Thomas, the architects of the "cancer immunosurveillance hypothesis", that "*there is little ground for optimism about cancer*" [6] and "*the greatest trouble with the idea of immunosurveillance is that it cannot be shown to exist in experimental animals*" [7], reflect the problems that, until recently, fomented intense debate over whether natural immune defense mechanisms can protect the host against the development of cancers of non-viral origin. The difficulty was clear: if immunosurveillance of developing tumors in immunocompetent hosts was indeed successful, then how could such an apparently invisible process be experimentally revealed? With the development of mouse tumor models using inbred mice with molecularly defined immunodeficiencies, the notion that the immune system intimately regulates cancer development experienced a new resurgence.

It is now recognized that the immune system plays at least three distinct roles in preventing cancer: (i) it protects the host against viral infection and hence suppresses virus-induced tumors; (ii) it prevents the establishment of an inflammatory environment that facilitates tumorigenesis by eliminating pathogens and by prompt resolution of inflammation; and (iii) it eliminates tumor cells in certain tissues because nascent transformed cells often co-express ligands for activating receptors on innate immune cells and tumor antigens that are recognized by immune receptors on lymphocytes of the adaptive immune system.

Nonetheless, tumors can and do arise in the presence of a functional and intact immune system. A troubled relationship exists between tumors and the immune system. Cancer cells lull immune cells into a false sense of security, thus avoiding immunosurveillance. The known ploys cancer uses are immunoselection (i.e., selection of non-immunogenic tumor-cell variants, a process also known as immunoediting) and immunosubversion (i.e., active suppression of the immune response) [3, 8, 9].

1.1.1 Cancer immunoediting: from immunosurveillance to tumor escape

Approximately 10 years ago, Schreiber and colleagues newfound the dual host-protective and tumor-promoting actions of immunity. In the face of continuous immune pressure, cancer cells can be shaped to become immunologically silent or refractory, and then better suited to survive ultimately causing harm. The acknowledgement that the immune system controls not only tumor quantity but also tumor quality (immunogenicity), has led to the refinement of the cancer immunosurveillance theory into one now termed cancer immunoediting [5].

In its most complex embodiment, the cancer immunoediting process is envisaged to proceed sequentially through three distinct phases: "elimination", "equilibrium", and "escape" (FIG. 1).

In the elimination phase, innate and adaptive immunity work together to destroy developing tumors long before they become clinically apparent. Many of the immune molecules (IFNγ; IFNαβ; IL-12; TNF; NKG2D; TRAIL; perforin) and cells (CD8 T, CD4 T, γδ T, NK, NKT, DC, Mϕ cells) that participate in the elimination phase have been identified, but more work is needed to determine their exact sequence of action. If this phase goes to completion, then the host

remains free of cancer, and elimination thus represents the full extent of the process. If, however, a rare cancer cell variant is not destroyed in the elimination phase, it may then enter the equilibrium phase, in which its outgrowth is prevented by immunologic mechanisms. T cells, IL-12, and IFNγ are required to maintain tumor cells in a state of functional dormancy, whereas NK cells and other effector cells or molecules are not required; this indicates that equilibrium is a function of adaptive immunity only. Editing of tumor immunogenicity occurs in the equilibrium phase. Equilibrium may also represent a second stable endpoint of cancer immunoediting and may restrain outgrowth of occult cancers for the lifetime of the host. However, as a consequence of constant immune selection pressure placed on genetically unstable tumor cells held in equilibrium, tumor cell variants may emerge that (i) are no longer recognized by adaptive immunity (antigen loss variants or tumor cells that develop defects in antigen processing or presentation); (ii) become insensitive to immune effector mechanisms; or (iii) induce an immunsuppressive state within the tumor microenvironment (immunosubversion) [3]. The end result is the generation via a Darwinian selection process of poorly immunogenic tumor cell variants that become "invisible" to the immune system and thus acquire the capacity to grow progressively and emerge in clinically apparent disease [5, 10].

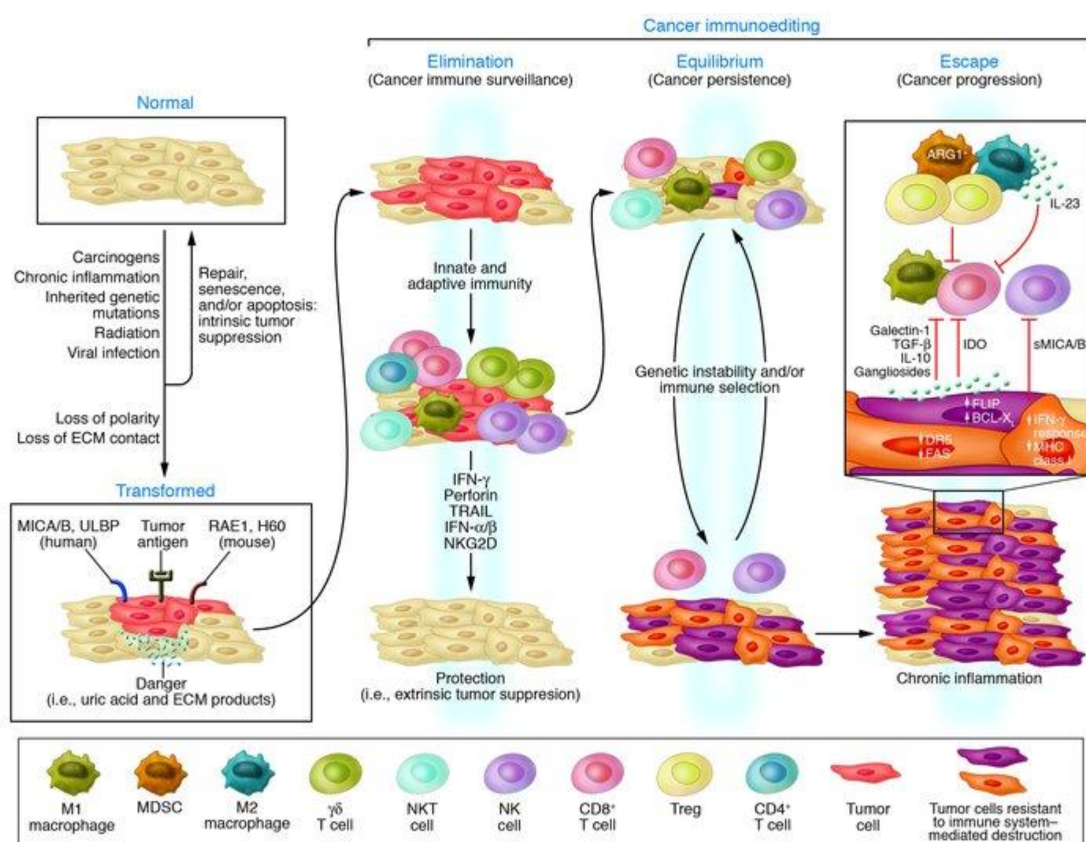

FIG.1 Extrinsic tumor suppression by the immune system. Transformed cells escaping intrinsic control are subjected to extrinsic tumor suppressor mechanisms that detect and eliminate developing tumors before they become clinically apparent. This is known as the elimination phase of a broader process that has been termed cancer immunoediting. Cancer immunoediting takes into account the observation that the immune system both protects the host against tumor development and promotes tumor growth. Cancer immunoediting is now considered a process composed of 3 phases: elimination, or cancer immune surveillance; equilibrium, a phase of tumor dormancy where tumor cells and immunity enter into a dynamic equilibrium that keeps tumor expansion in check; and escape, where tumor cells emerge that either display reduced immunogenicities or engage a large number of possible immunosuppressive mechanisms to attenuate antitumor immune responses leading to the appearance of progressively growing tumors. These phases have been termed the 3 Es of cancer immunoediting. (*Figure adapted from Swann JB and Smyth M, The Journal of Clinical Investigation, 2007*)

1.1.2 Immunosubversion

The molecular tricks by which tumor cells can subvert the immune system thus 'paralyzing' immunosurveillance are the subject of intense investigation.
It was originally thought that the inefficiency of tumor-associated antigen (TAA)-specific immunity was due to intrinsic causes: (i) tumors simply did not present enough TAA; (ii) antigen-presenting cells (APC) did not have sufficient stimulatory capacity; or (iii) there were not enough effector cells or effector cytokines. On this basis, attempts were made to bolster TAA-specific immunity through administration of stimulatory cytokines (IL-2, IL-12 or IFNα) or TAA (peptides), or by using optimal APC (DC vaccines) [11-20]. In a different approach, TAA-specific effector T cells from cancer patients were expanded *ex vivo* followed by adoptive transfer [13, 16, 21, 22]. These approaches were met with some success both in mouse models and in early clinical trials in humans, thus strengthening experimentally induced TAA-specific immunity as an efficacious approach to treat established tumors.

Recent studies have shown the other side of the coin: poor TAA-specific immunity is not due to a passive process whereby adaptive immunity is shielded from detecting TAA. On the contrary, there is an active process of "tolerization" taking place in the tumor microenvironment [23]. In mouse models, advanced cancer invariably subverts immune function. Typically, tumor-specific CD8 T cells are activated at the stage of initiation of tumor growth, but these cells show a progressive loss of cytolytic function at the later stage of tumor expansion [24]. Similarly, tumor-specific CD4 T cells progressively lose their antitumor activity [25], whereas the number of regulatory T (Treg) cells increases. One possible explanation for how tumors subvert the immune response is to consider that the tumor is a "false lymphoid organ; therefore, T-cell priming in the tumor microenvironment is defective as a result of the presence of dysfunctional or tolerogenic antigen-presenting cells. Indeed, some tumors overproduce various factors (such as vascular endothelia growth factor (VEGF), IL-6, IL-10, transforming growth factor (TGF)β, macrophage colony-stimulating factor (M-CSF), nitric oxide synthase (NOS)2, arginase-1, indoleamine 2,3 dioxygenase (IDO), prostaglandin (PG)E2, cyclooxygenase (COX)2 and gangliosides that can inhibit the differentiation, maturation and function of DC [26] as well as T-cell function [27]. Accordingly, local DC tend to mediate immunosuppressive, rather than immunostimulatory, effects and to promote IL-10 producing Treg-cell differentiation [26, 28]. Moreover, some human tumors (prostate, colon and pancreatic carcinomas) constitutively express IDO assigned for tryptophan degradation. This metabolic device blocks local proliferation of CD8 T cells [29] and promotes apoptosis of CD4 T cells, thus promoting resistance to immune-mediated rejection. Yet some other tumors can express CD95L still killing CD95-expressing tumor-specific T cells [30].

A series of recent studies have proposed new disadvantageous leukocytes to add to the list of suppressive cells to challenge. In an ultraviolet-irradiation-induced tumor model, irradiation-induced immunosuppression was found to be mediated by CD1d-restricted natural killer T (NKT) cells. These CD4 NKT cells produced IL-13, which suppressed CTL-mediated tumor rejection. Moreover, IL-13 from NKT cells activated myeloid suppressor cells to produce TGFβ, which also suppressed cytotoxic T lymphocytes (CTL) activity [31]. In addition, tumor-associated macrophages (TAM) mostly belong to the M2 class of macrophages, fully polarized to produce arginase-1, IL-10, TGFβ and PGE2, thus playing a key role in subversion of adaptive immunity and in inflammatory circuits that promote tumor growth and progression [32, 33] . Another possible explanation for tumor-mediated immunosubversion is based on a quantitative issue. Cancer traits that are immunostimulatory in small tumors can become immunosuppressive in large tumors. For example, the expression of NKG2D ligands (which stimulates an immune response at the initial stages of oncogenesis, as discussed earlier) seems to be immunosuppressive in larger tumors. NKG2D-ligand expressing tumor cells (as well as soluble NKG2D ligands that are shed from tumor cells) can downregulate NKG2D expression by CD8 T cells and NK cells or can uncouple NKG2D signaling from intracellular mobilization of Ca^{2+} or cell-mediated cytolysis, thereby contributing to suppression of the immune response [34]. Similarly, it could be argued that large tumors cause a general or specific downregulation of T-cell responses as a result of "high-dose tolerance" to TAA. Following successful systemic chemotherapy — for example, for ovarian carcinoma — CD8 T-cell function can recover [35], indicating that antitumor chemotherapies that have limited immunosuppressive side-effects can restore the normal immune response by abolishing tumor-mass-related immunosubversion.

Although "black and white" signals have been identified in tumor immunity, from what described above, it is evident that this is an oversimplification and that interactions between tumor cells and immune cells would be represented by a multitude of colors. So, the "bad news" is that cancer cells can strategically avoid immune attack. The "good news" is that this newfound knowledge, comprehensive on the tumor and the immune status, is a powerful tool to which oncologists might capitalize aiming to the optimal management of the disease. As stated by Prendergast and Jaffee, to win the fight against cancer is necessary to stop "*segregating cancer immunology from cancer genetics and cell biology*" [36].

1.2 CHEMOTHERAPY AND TUMOR IMMUNITY: AN UNEXPECTED COLLABORATION

Cancer therapy is continuously evolving in order to strategically optimize the chance of cure. The therapeutic approach to cancer today most frequently involves surgery (whenever possible) alone or in association with a single-agent or combinatorial treatment based on radio- or chemotherapy. Radiation therapy is used to achieve locoregional control, whereas systemic therapies (chemotherapy, endocrine therapy, molecularly targeted therapies, and adjunctive therapies – bisphosphonates -) are used to control diffuse disease (in hematologic malignancies) or disease that has spread beyond the primary site (in solid tumors).

A growing body of evidence suggests that conventional therapy for cancer may profit from the participation of the immune system whose contribution is elicited in two ways. On one hand, some therapeutic programs can tickle specific cellular responses — beyond the stereotypical apoptotic pathway — that render tumor-cell death immunogenic. On the other hand, some drugs may have side effects (beyond their effect on the tumor itself) that stimulate the immune system, through a transient lymphodepletion, the subversion of immunosuppressive mechanisms and the direct or indirect stimulatory effects of immune effectors. Moreover, vaccination against cancer-specific antigens can sensitize the tumor to subsequent chemotherapeutic treatment.

1.2.1 Immunogenic cancer cell stress and death

It has been generally assumed that most if not all chemotherapeutic agents induce cancer cell death by apoptosis and that apoptotic cell death would - by definition - lead to silent corpse removal and hence fail to induce an immune response against the dying cells. In apparent contrast with this idea, some chemotherapeutic agents do induce a type of cell death that is immunogenic, yet is accompanied by the all known biochemical and morphological hallmarks of apoptosis. Thus, tumor cells that have been killed *in vitro* with some chemotherapeutic agents such as anthracyclines, oxaliplatin or cyclophosphamide [3, 37, 38] (but not with others such as cisplatin) elicit a tumor-specific cytotoxic T lymphocyte response when they are injected subcutaneously into immunocompetent mice. This leads to the long-term protection of vaccinated mice against challenge with live tumor cells of the same type. In essence, a limited array of antineoplastic drugs induces immunogenic cancer cell death (ICD), which in turn provokes an anticancer immune response that allows the immune system to control (and possibly to eliminate) residual tumor cells. Such tumor-host productive dialogue involves the transfer of TAA to immune cells that stimulate a tumor-specific immune response. This is critical for the eradication of residual cancer (stem) cells as it operates irrespective of their resistance to therapy offering a possible explanation to how the anticancer immune response can contribute to the undeniable success of some antineoplastic regimens.

1.2.1.1 *The key-lock paradigm*

Cancer cells dying in an immunogenic fashion emit specific cell death-associated molecular patterns (CDAMP) that - in a correct spatial and temporal appearance - bear the ability to convert non-immunogenic corpse removal into an immunogenic reaction. Obviously, such a conversion also relies on the correct perception of CDAMP by dedicated sentinels of the host immune system. Thus, antigens from cancer cells succumbing to ICD inducers (like anthracyclines, oxaliplatin, cyclophosphamide and ionizing radiations) are efficiently taken up and processed by DC, which in turn cross-prime naïve T cells and drive the development of a tumor-specific immune response. The interaction between DC and dying cancer cells is controlled by the emission and/or release from the latter of the so-called "eat me" and "don't eat me" signals, i.e., membrane-bound or soluble molecules that stimulate or inhibit phagocytosis, respectively. The systematic analysis of surface proteome alterations in anthracycline-treated tumor cells revealed that ICD is associated with the ectopic co-exposure of the endoplasmic reticulum (ER) chaperones calreticulin (CRT) and ERp57 [39]. Ecto-CRT functions as an "eat-me" signal for DC, thereby facilitating DC-mediated antigen uptake, and is an absolute requirement for the immunogenicity of dying tumor cells [39]. The co-exposure of CRT and ERp57 reportedly ensues the induction of an ER stress response that is associated with massive ultrastructural alterations of this organelle, and depends on the activation of (at least) three signaling modules. First, the ER-resident protein kinase R-like endoplasmic reticulum kinase (PERK) gets activated and couples ER stress signals to translation inhibition by phosphorylating the eukaryotic initiation factor 2α (eIF2α). Accordingly, the disruption of the eIF2α phosphatase complex PP1/GADD34 by small peptide inhibitors, resulting in increased phospho-eIF2α suffices to trigger CRT exposure in cancer cells [38, 40]. Second, an apoptotic module that involve the mitochondrion-permeabilizing proteins BAX and BAK (which also work at the interface between the ER and mitochondria to regulate calcium fluxes) [41, 42], caspase-8 and its substrate BAP31 - an ER sessile protein implicated in the lethal response to ER stress - is activated. Thus, the pan-caspase inhibitor Z-VAD-fmk, as well as genetic interventions whereby BAX, BAK and/or caspase-8 are removed or depleted, blocks CRT exposure and abolishes the tumor-vaccinating effect of cells undergoing ICD [39, 40]. Third, approximately 5-10 % of the endogenous CRT pool is exposed together with ERp57 at the surface of dying cells via SNAP and NSF attachment receptor (SNARE)-dependent exocytosis. This occurs well before plasma membrane permeabilization (which occurs as the final step of apoptosis), and also precedes the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. PS is the

prototypic "eat-me" signal of apoptotic cells (though it has been implicated also in non-apoptotic cell death) [43] and the kinetics of its exposure might affect the switch between the silent removal of dying cells by macrophages and the initiation of a cognate immune response by DC. The receptor that is responsible for antigen uptake by DC upon CRT binding remains to be determined. Possible candidates include the major CRT receptor CD91 as well as other CRT-interacting proteins like scavenger receptor A (SR-A), scavenger receptor expressed on endothelia cell I (SREC-I), CD40 ligand, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or CD95/FAS ligand. The CRT-driven uptake of tumor antigens by DC is *per se* insufficient to elicit an antitumor immune response as internalized antigens must be processed and re-exposed for the cross-priming of CD4 and CD8 T lymphocytes. This implies that other signaling pathways are involved in ICD.

A systematic study of the response to CDAMP of distinct Toll-like receptors (TLR) on naïve T-cells revealed that TLR4 is both required and sufficient for efficient antigen presentation by DC [44]. Among other proteins, TLR4 binds the non-histone chromatin-binding nuclear protein high-mobility group box 1 (HMGB1), leading to the activation of the downstream effector myeloid differentiation primary response 88 (MYD88). This inhibits the fusion between lysosomes and antigen-containing phagosomes, thus facilitating antigen processing and presentation to T cells. HMGB1 also stimulates the neo-synthesis of pro-IL-1β but *per se* does not serve as a DC maturation signal. The release of HMGB1 from tumor cells succumbing to ICD manifests with a dual kinetics whereby HMGB1 first translocates from the nucleus to the cytoplasm and then, following the breakdown of the plasma membrane, gets released into the extracellular space [45].

The vaccine-like effect of ICD relies on the elicitation of an IFNγ-polarized T cell response, which in turn requires the function of the NLRP3 inflammasome, a multiprotein caspase-1-activating complex. Caspase-1 activation is critical for activating an antitumor immune response as it catalyzes the proteolytic maturation of IL-1β [46]. One of the most abundant factors that activate the NLRP3 inflammasome is ATP, and at least in DC, it does so by binding to the purinergic P2RX7 receptor on the cell surface. ATP also constitutes a CDAMP, as it gets released during the final steps of cell death, possibly via voltage-gated hemichannels of the pannexin 1 or connexin type [47]. Accordingly, the depletion of intracellular or extracellular ATP in cells succumbing to ICD abolishes the development of an IFNγ-polarized response, and P2RX7-deficient mice fail to mount an immune response against syngeneic cancer cells succumbing to ICD. Intriguingly, ATP also serves as a "find me" signal for the attraction of immune cells. Altogether, these observations highlight the multifaceted and critical role of ATP for the vaccine-like effects of ICD inducers.

The spatially and temporally regulated emission of immunogenic factors from dying tumor cells accounts for the recruitment and activation of immune cells to tumor bed and governs the immune response to cancer cells undergoing ICD. Thus, the stress conditions that cancer cells confront during chemo- and radiotherapy determine whether the subsequent wave of cell death will elicit an antitumor immune response or rather will remain immunologically silent. Normally, cells attempt to cope with stress by arresting normal activities and by activating a series of cytoprotective mechanisms that aim at re-establishing homeostasis. This is accompanied by alterations of the surface proteome that, in the case of ICD, account for the recognition by immune cells, and by the emission of soluble mediators with chemotactic and anti-chemotactic properties. This is crucial for the "selection" and differentiation/maturation of engulfing cells, which in turn dictates the immunogenic or tolerogenic outcome of cell death. In this sense, the exposure of the DC-specific "eat me" signal CRT paralleled by the disclosure of other, hitherto uncharacterized, "don't eat me" signals, facilitates the recognition and uptake of dying tumor cells by DC rather than by macrophages. Based on these observations, the spatially-restricted and temporally-ordered appearance of CRT, HMGB1 and ATP might constitute a "key" that would precisely fit into a series of pattern recognition receptors (PRR) expressed by DC (the "lock") for the conversion of non-immunogenic into immunogenic cell death and for the elicitation of an anticancer immune response [45] (FIG.2).

By shaping T cell responses, DC are the first-line decision makers of the innate immune system and their role in immunogenic chemotherapy has been deeply investigated. Experiments in transgenic mice that express the diphtheria toxin receptor (DTR) under the control of a DC-specific promoter (allowing for *in vivo* DC depletion) revealed the essential role of DC in the perception and decoding of "come and get-me" signals emitted by dying tumor cells during ICD [44]. Similarly, the *in vivo* depletion of CD8 T cells with specific antibodies has been instrumental to highlight the critical role of this lymphocyte subset for the vaccine-like effect of chemo- and radiotherapy in a large panel of murine tumor models, including CT26 colon cancer, EL4 thymomas, TS/A mammary carcinomas, MCA205 fibrosarcomas and GOS osteosarcomas. In line with these observations, CD8 T cells have been shown to mediate potent anticancer immune effects in clinical settings, for instance in colorectal tumors, where immune infiltration might serve as a prognostic factor [48]. Moreover, it has recently been shown that a precise orchestration of the T cell response is required for immune effectors to eradicate tumors. In this context, the IL-1β-dependent recruitment of IL-17-secreting γ/δ T cells had to precede the infiltration of tumors by Tc1 lymphocytes for the efficacy of immunogenic chemotherapy *in vivo* [49]. Thus, a finely regulated crosstalk between components of the innate (DC) and cognate (γ/δ and CD8 T cells) immune system is required for cell death to be perceived as immunogenic, for the elicitation of an anticancer immune response, and for complete tumor eradication leading to therapeutic success.

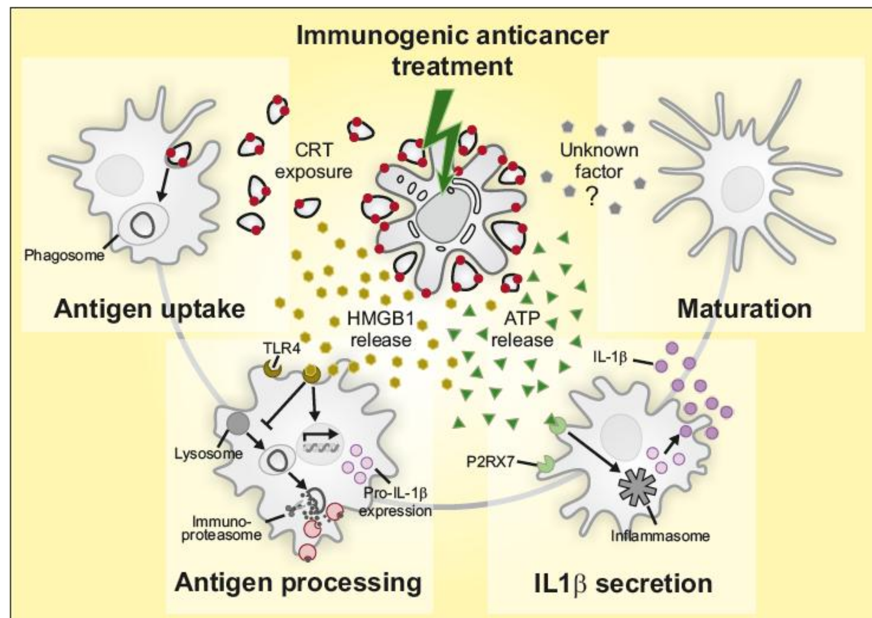

FIG.2 Immunogenic signals emitted by dying cells form a spatiotemporal code unlocking DC to mount a potent immune response toward tumor cells. (i) Early exposure of ecto-CRT by dying tumor cells, which facilitates engulfment by DC. (ii) HMGB1 released from dying cells binds to TLR4 on DC, thus favoring antigen cross-presentation and up-regulating pro-IL-1β. (iii) ATP liberated from dying cells binds to the purinergic receptor P2RX7 on DC, activates the NLRP3 inflammasome, and leads to the secretion of active IL-1β, which polarizes CD8+ T cells toward IFN-γ production. (iv) An additive DC maturation factor remains to be characterized. (*Figure adapted from Hannani D et al.,Cancer J, 2011*)

1.2.2 Immunostimulatory side effects of anticancer drugs: Cyclophosphamide as elected drug

Cytotoxic drugs can be used in unexpected ways to break systemic mechanisms of immune tolerance, or to alter the host environment in which the antitumor immune response develops.

Many chemotherapy drugs can have both positive and negative immunomodulating activity, with the type of influence depending on the drug dosing and the relative timing of administration [50].
Cyclophosphamide (CTX), the lead compound of alkylating agents, is one of the most potent and widely used drugs for the treatment of hematological and solid organ malignances, autoimmune disorders, and as a conditioning regimen for blood and marrow transplantation.

In order to achieve cytotoxic effects, CTX needs to be metabolically converted by the hepatic oxidase into its metabolites acrolein and phosphoramide mustard which are responsible for the cytostatic and urotoxic effects of the drug, respectively, through DNA-alkylating and cross-linking mechanisms. The metabolic activation of CTX is irreversibly compromised in cells with high levels of aldehyde dehydrogenase 1 (for example, hematopoietic stem cells). This enzyme converts aldophosphamide into carboxyphosphamide. Carboxyphosphamide does not decompose to phosphoramide mustard and therefore lacks alkylating and cytotoxic activity (FIG. 3).

Depending on the dosage and the timing of administration, CTX displays either immunosuppressive or immunopotentiating effects [51]. At high dosage, CTX exerts potent cytotoxic and lymphoablative effects, indispensable for dose intensity and immunosuppressive regimens in the oncological and internal medicine armamentarium. More recent work highlighted the immunostimulatory and antiangiogenic effects of low dosing or metronomic (i.e., frequent, repeated low doses) CTX, thus repositioning this drug in the field of cancer immunotherapy.

It is interesting to note that a single CTX injection induces a transient myelo-lymphodepletion (whose intensity depends on the dose) that is followed by a recovery phase during which homeostatic mechanisms occur increasing leucocyte cell counts well above baseline levels. Following this “rebound” event, cell counts slowly reach pre-treatment levels (FIG.4). This double-face activity accounts for many of the observed immunomodulatory effects ascribed to the drug and for the capability of synergizing with a series of immunotherapy approaches.

Great relevance was attributed to the reduction of cells that negatively modulate immune responses *in vivo*. Studies performed by Awwad and North in the 80s demonstrated that CTX administration can enhance the effectiveness of adoptive immunotherapy through the selective reduction of a suppressor T cell population, that was not clearly identified at that time [52]. Later work from Ghiringhelli’s and Lutsiak‘s groups showed, indeed, that the administration of CTX reduces the number [53] and inhibits the functions of CD4 CD25Tregs in tumor-bearing animals by

downregulating the expression of the key functional markers forkhead box P3 (FOXP3) and glucocorticoid-induced TNF-receptor-related protein (GITR) [54]. This was a formal proof of how CTX treatment circumvents tumor-induced immune tolerance tipping the balance toward an effective antitumor response. Furthermore, mitigating the influence of Treg and stimulating IFNα/β production by host leukocytes [55], CTX might account for the augmented antibody responses and the persistence of memory T cells. All these effects contribute to the eradication of immunogenic tumors in synergy with specific immunotherapy approaches [56, 57]. As reported by Ercolini and colleagues, CTX pretreatment affects Treg/Teffector ratio enabling the vaccine-mediated recruitment of high avidity CD8 T cells to the antitumor response in tolerized *neu* transgenic mice [58]. Importantly, these findings correlate with tumor rejection, an outcome never seen with vaccine alone in this tolerized setting.

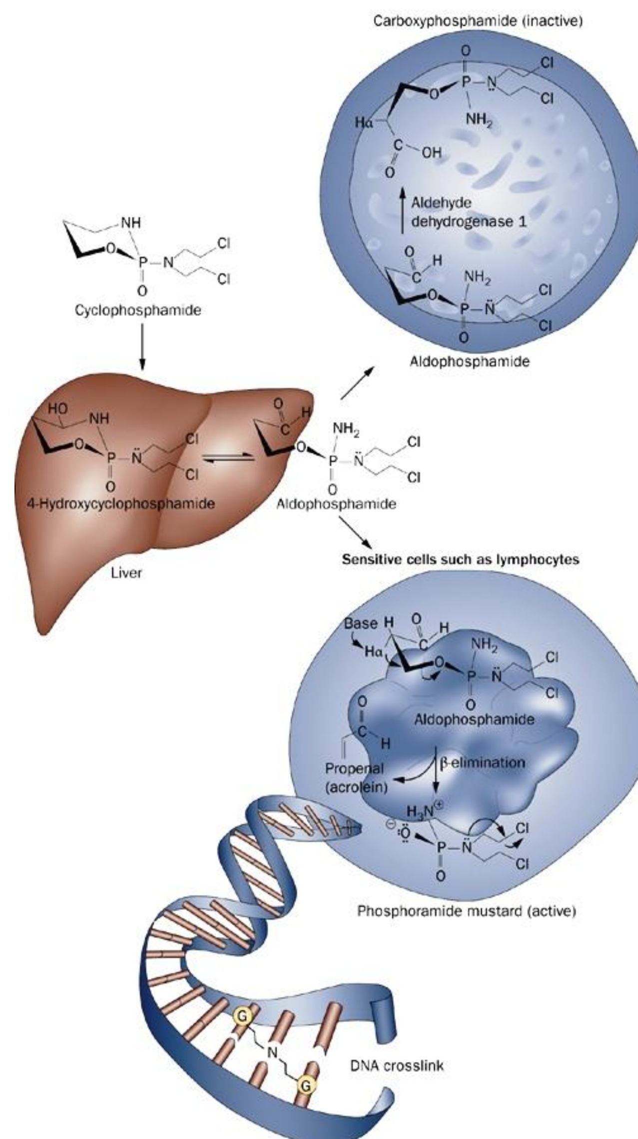

FIG. 3 Cyclophosphamide metabolism. After intravenous or oral administration, cyclophosphamide is rapidly distributed in the body. In the liver, it is converted to 4-hydroxycyclophosphamide, which stays in equilibrium with aldophosphamide. 4-hydroxycyclophosphamide and aldophosphamide readily cross the cell membranes by passive diffusion. In cells with high levels of aldehyde dehydrogenase 1 (for example, hematopoietic stem cells), aldophosphamide is irreversibly converted to carboxyphosphamide. Carboxyphosphamide does not decompose to phosphoramide mustard and therefore lacks alkylating and cytotoxic activity. In the absence of a high concentration of aldehyde dehydrogenase (for example, lymphocytes), aldophosphamide spontaneously liberates phosphoramide mustard and acrolein. Phosphoramide mustard is a bifunctional DNA alkylating molecule and forms interstrand DNA crosslinks primarily at the guanine (G) sites. (*Figure adapted from Emadi A et al., Nature Reviews, 2009*)

In small clinical studies, the combination of low doses of intravenous CTX with vaccines has been shown to augment delayed type hypersensitivity (DTH) responses [59, 60], decrease the proportion of suppressor T cells [61] and prolong the survival of patients with metastatic cancer [59]. One month of metronomic program of CTX given to patients with end-stage cancer could suppress Treg-cell inhibitory functions, restore the proliferative capacity of effector T cells and restore the cytotoxicity of NK cells [62, 63]. However, the ablation of regulatory cells is likely to be of varying importance, depending on the tumor type, stage and location [64]. Further studies revealed that the strong therapeutic efficacy of combined chemoimmunotherapy stems from a bystander effect on host lymphocytes (as well as adoptive lymphocytes) occurring during the recovery phase following CTX-induced myelo/lymphodepletion [22, 65]. An early study from Proietti's group demonstrated that a single injection of CTX, followed by the adoptive transfer of antitumor immune cells, could eradicate established tumors and prevent metastases spreading. This effect depended on the production of a plethora of until then uncharacterized soluble factors, which may sustain the proliferation, survival and activity of transferred cells [65]. Of note, such antitumor efficacy was abolished when mice were treated with antibodies neutralizing type I IFN. A subsequent study clarified that CTX leads to the expression of type I IFN *in vivo*. Among the soluble factors coming out in the so called "cytokine storm", type I IFN are obligate for the expansion of CD4 and CD8 T cells exhibiting a memory (CD44^{hi}) phenotype [55]. CTX role in favoring memory T cells was later confirmed by the finding that prolonged metronomic schedule of chemotherapy, despite diminishing the number of proliferating tumor-specific CTL, preserved CD43^{low} memory CD8 T cells [66].

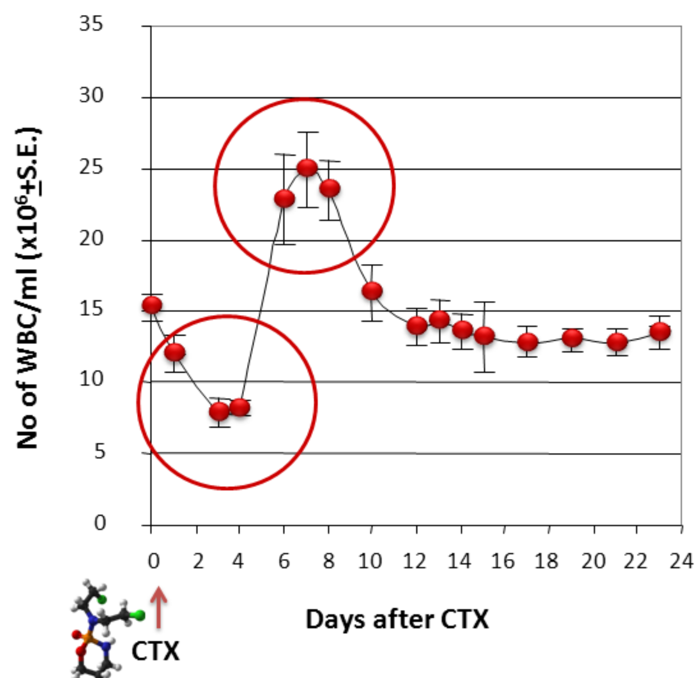

FIG. 4 After CTX (100 mg/kg)-induced lymphodepletion, a "rebound" phase occurs, during which a cytokine storm drives the homeostatic proliferation, activation and trafficking of different lymphocyte pools. (*Figure adapted from Proietti E et al., J Clinl Invest, 1998)*

More recent studies showed that CD4 T cells are responsible for the synergism between chemotherapy and adoptive immunotherapy and for the cooperation of transferred cells with the host immune system [67]. Furthermore, CTX promoted the migration of specific tumor-immune lymphocytes to the tumor bed and induced the homeostatic proliferation/activation of transferred B and T lymphocytes. A first characterization of the molecular mechanism underlying the immunomodulatory properties of CTX came from the gene expression analysis of a selected panel of cytokines by real-time PCR in the bone marrow (BM) and spleen of treated mice. Optimal therapeutic responses to the adoptive transfer of immune cells were found to be associated with the chemotherapy-mediated induction of a "cytokine storm" occurring during the rebound phase after drug-induced myelo-lymphodepletion [67]. In a subsequent study, the transient upregulation of a variety of immunomodulatory factors, including danger signals, pattern recognition receptors, inflammatory mediators, growth factors, Th1-polarizing and homeostatic cytokines, chemokines, and chemokine receptors was observed by gene and protein expression analysis early after CTX injection [68]. These

factors are involved in sensing CTX myelotoxicity and activating repair mechanisms, which, in turn, stimulate immunoactivation events that promote chemotherapy efficacy. Notably, the relevance of the increased expression of homeostatic cytokines (IL-7 and IL-15) was also confirmed by the finding that the antitumor efficacy of the combination of a subletal total body irradiation (TBI) with the adoptive transfer of CD8 T cells was impaired in mice deficient of both cytokines [69]. Of great interest, Matar and colleagues observed a Th2 to Th1 shift in cytokine production in a rat metastatic lymphoma setting after treatment with low-dose of chemotherapy [70]. In line with these observations, it has been demonstrated that low-dose, as well as metronomic CTX, promotes the expansion and differentiation of CD4 T producing IL-17A, in naïve and tumor bearing mice [68, 71]. These data agreed with the clinical observation of advanced cancer patients treated with non myeloablative and non lymphodepleting doses of CTX (3 week-oral treatment with 50 mg/day). *Ex vivo* IL-17 release by circulating peripheral blood mononuclear cells (PBMC) after T cell receptor (TCR) stimulation (anti-CD3/CD28 Ab cross-linking) were significantly enhanced following CTX regimen [71] .

An increasing number of studies underscored that also the innate arm of the immune response might be involved in the immunopotentiating activity of chemotherapy. According to Salem and colleagues, systemic CTX increases the relative number and the activation status of myeloid DC through the induction of high levels of inflammatory cytokines such as IFNα and IL-6 among others [72]. In accordance, Radojcic and colleagues found that a myelosuppressive dosage of CTX, perturbs DC homeostasis. This leads to the occurrence of tumor-infiltrating DC locally secreting IL-12, and therefore able to prime T-cell responses [73] (FIG.5).

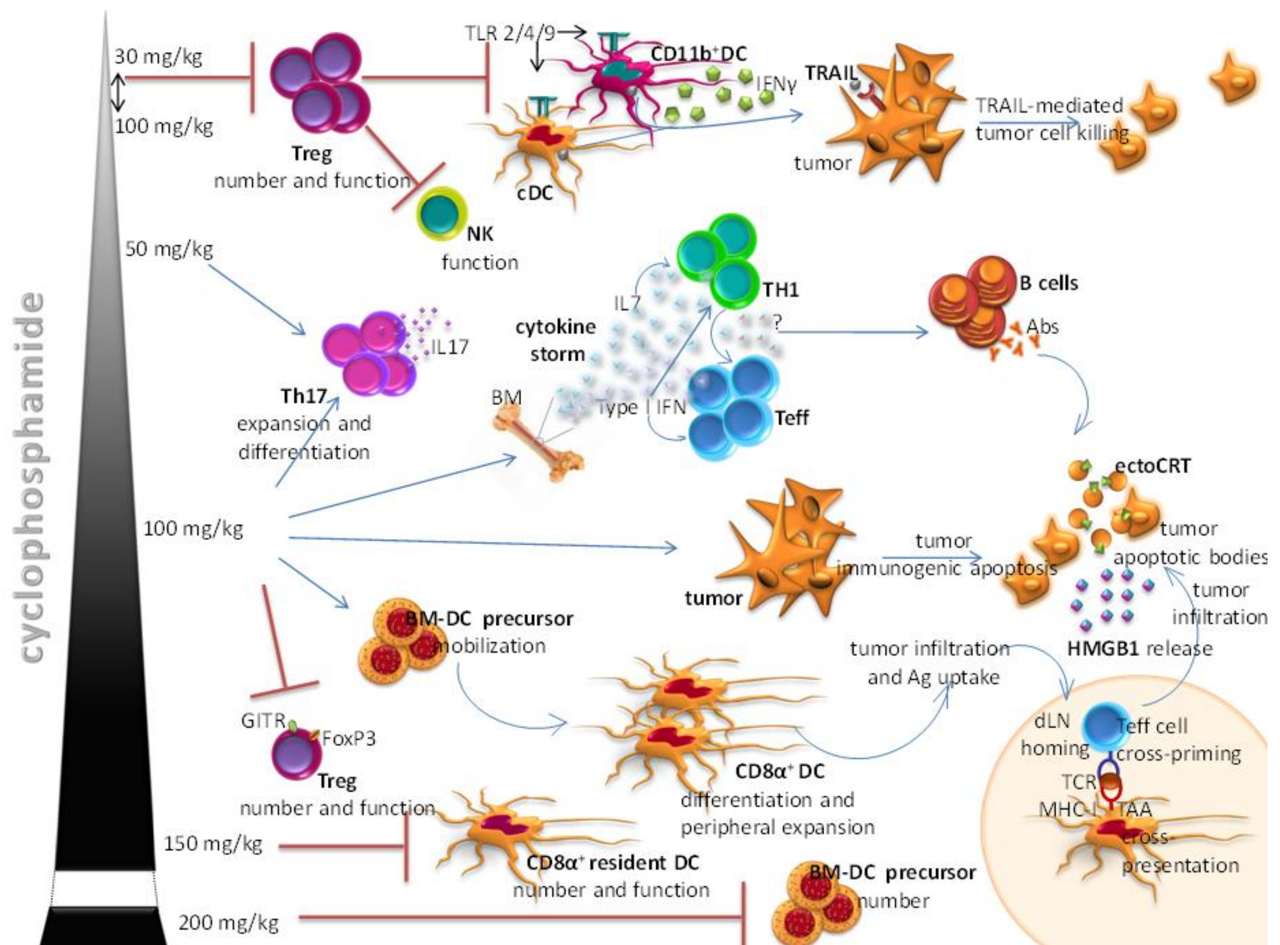

FIG.5 Immunomodulatory effects of cyclophosphamide. (*Figure adapted from Sistigu A et al.,Semin. Immunopath., 2011*)

Fifty years after the US Food and Drug Administration (FDA) approval, CTX remains a safe and affordable compound endowed with multifaceted properties and a plethora of clinical indications. The acknowledgement of chemotherapy -in general- and CTX -in particular- immunomodulatory effects strengthens the need for a rational

combination of chemo- and immune- therapy, leveraging additive or even synergistic activity. The thoughtful combination of multiple treatment modalities should allow the full power of immunotherapy to be unleashed, resulting in increasing survival benefits and ultimately in the eradication and relapse prevention of malignant disease.

1.3 IMMUNOTHERAPY

Cancer immunotherapy consists of approaches designed to reconstruct host-tumor immunobiology, tipping the balance of immunologic homeostasis in favor of the host.

The concept of cancer immunotherapy goes back as far as the late nineteenth century, when Coley observed tumor shrinkage and even disappearance following the injection of bacterial products in and around tumors [74]. Since then, many observations, such as the rare but well-documented occurrence of spontaneous remissions, the higher incidence of cancer in patients who are immunosuppressed, and the identification of tumor-specific antigens and lymphocytes, have stimulated research on strategies that aim to induce specific antitumor responses. Over the past decades, considerable knowledge has been obtained on the components that are relevant in antitumor immune responses and immune escape mechanisms, yet the development of immunotherapy as a treatment modality for cancer has been hampered by several factors. These include difficulties in the selection of the optimal dose and schedule, the methods of evaluation, and financial support. Although durable clinical remissions have been observed with various immunotherapeutic strategies, the percentage of patients who benefited from these interventions has remained too small to justify the general use of such strategies. As a consequence, for many years, the clinical progress in the field of immunotherapy has been slow. However, the recent positive preclinical and clinical results with novel immunoactive drugs as well as the unexpected finding of a positive interaction between immunotherapy and chemotherapy may herald a new era for the immunotherapy of cancer. An additional great boost to immune-based therapy derived from improved ways of evaluating responses to treatment due to the progressive understanding of immunotherapy-induced responses.

Of great importance, immunotherapy is uniquely able to exert a durable therapeutic effect due to the induction of immunologic memory, minimizing systemic toxicity. This effect makes prolonged, repetitive cycles of therapy unnecessary and identifies immunotherapy as a promising modality for the secondary prevention of disease relapse and ultimately the prevention of primary tumor development.

Immune-based therapy can be broadly divided into approaches that employ the passive administration of immunologic effectors like monoclonal antibodies (mAb), cytokines and lymphocytes, and strategies that actively induce these immune effectors *in vivo* (i.e., vaccines).

1.3.1 Cancer Vaccines

Prevention or treatment with a cancer vaccine, or active specific immunotherapy, is a very attractive therapeutic option because the mechanism of action is eventually an enhanced endogenous immune response against the host's malignancy [50, 75]. Vaccine approaches utilize tumor antigens and antigen-presenting cells to enhance a preexisting antitumor immune response, or, perhaps in some cases, to induce an antitumor immune response that did not previously exist. There are many potential sources of tumor antigens including purified or synthesized tumor-cell surface molecules, which may be peptides or proteins, cells or lysates derived from fresh or cryopreserved autologous tumor samples (which is actually a mixture of normal and malignant cells), and cells or lysates of allogeneic or autologous tumor cell lines. There are a variety of methods by which tumor antigens can be presented including a purified antigen, via heat shock proteins, in viruses, or DNA, or by APC such as DC, or as the idiotypes of mAb that have been selected by their tumor antigen recognition. There are numerous molecules that might be useful as adjuvants to enhance the immunogenicity of a vaccine. There are also many routes by which vaccinations might be delivered including subcutaneous, intradermal, intramuscular, intravenous and intralymphatic.

As vaccines are minimally associated with side effects and invasive procedures, it is time to consider whether they can also be used to prevent tumor development. As microorganisms are the cause of 10-20% of all human tumors (reported in *World Cancer Report – Stewart BW & Kleihues P – IARC Press, Lyon 2003*), vaccines that reduce infection with viruses that cause cancer are of the utmost importance in primary cancer prevention. Vaccination against hepatitis B virus, for example, has reduced the incidence of hepatocellular carcinoma [76], whereas vaccines against human papilloma viruses are expected to greatly reduce the incidence of cervical carcinoma [77].

In animals, anti-tumor vaccines are effective in preventing a subsequent tumor challenge. This is a well-substantiated observation established through countless tumor-challenge experiments performed in immunized animals using many different fast-growing and aggressive mouse tumors [78]. In these experiments, immunization against a tumor antigen is followed by a subcutaneous, intramuscular or orthotopic challenge with a lethal dose of a transplantable tumor. In mice, effective immunity is often elicited and a successful pre-immunization against almost any kind of tumor seems to be feasible.

Unfortunately, there are usually several etiological agents for most human cancers and therefore instead of being prophylactic, vaccine strategies need to be therapeutic. In 2005, more than 200 clinical trials were in progress (reported in *ClinicalTrials.Gov database of the National Cancer Institute & European Organization for the Research*

and Treatment of Cancer Protocols Database websites). The results achieved so far, however, have been poor: partial responses were rare and complete responses extremely rare. Only in few patients has the progression of previously growing tumors been halted and prolonged survivals observed. As benefits that were due to vaccination have been sustained in no more than a handful of cases [79], new strategies are being explored [12, 79], including vaccines based on engineered viral vectors, various approaches with DC, and strategies that are aimed at inhibiting immunosuppressive cells of lymphoid or myeloid origin. Perhaps vaccination alone is not the solution for treating existing tumors, and evidence is emerging that shows that combining immunotherapy with chemotherapy, radiotherapy, anti-angiogenic therapy and other approaches could yield synergistic or additive results [80, 81]. Therapeutic vaccines are also poorly effective in mouse models of cancer, so the lack of benefit seen in clinical trials is perhaps not surprising. More knowledge on the schedules, routes, doses and adjuvants is required to optimally use these strategies. However after many years of hard work and negative trials, it was gratifying when, in 2010, the cell product Sipuleucel-T emerged as the first therapeutic vaccine approved by the US FDA for the treatment of prostate cancer. This cell-based vaccine consists of autologous PBMC, which include professional APC that have been activated with a fusion protein of the prostate antigen prostatic acid phosphatase and the immunostimulant GM-CSF. Sipuleucel-T was approved based on results from a placebo-controlled Phase III randomized trial. Despite showing a lack of benefit in progression-free survival and the fact that tumor regressions were rare, an overall survival benefit of 4.1 months was demonstrated compared to placebo [82]. Other examples of promising vaccine-based approaches have now entered Phase III clinical trials include an idiotype-based vaccination plus GM-CSF for follicular lymphoma, a combination of gp100 and high dose IL-2 for melanoma [83], a prostate-specific antigen-targeted poxviral vaccine in prostate cancer [84], and a melanoma-associated antigen-3-protein vaccine in non-small cell lung cancer [85].

It is expected that predictive oncology will assess the individual risk of cancer as a function of sex, age, family history, genetic makeup and lifestyle (reported in *American Cancer Society Who is at Risk & your Disease Risk websites*), whereas gene expression profiles and molecular biology will outline the probability that a particular onco-antigen will be expressed by the tumor for which the person is at risk. By combining this information, one can envisage to appeal to a custom-tailored preventive vaccination.

1.3.2 Monoclonal Antibodies

The concept of using antibodies to selectively target tumors was proposed by Ehrlich over a century ago (reported in *Ehrlich, Collected studies on immunity. New York: J. Wiley & Sons, 1906*).

The advent of hybridoma technology in 1975 enabled the production of immunoglobulins from a single clone of B-cells, and hence the term “monoclonal antibodies” (mAb) [86]. The potential clinical application of such biological products in cancer therapy was quickly recognized and repeatedly emphasized [87-89]. However their applicability in the field of immunotherapy has been slow. Owing to their origins in mice, mAb were typically immunogenic in humans and had poor abilities to induce human immune effector responses. Later advances in antibody engineering provided flexible platforms for the development of chimeric, humanized and fully human mAb which satisfactorily addressed many of these problems (reported in *Dillman, Principles of cancer biotherapy, 2009*). The past decades have witnessed the evolution of mAb as ‘magic bullets’ from concept to clinical reality [90], and their effectiveness in treating cancer patients has been increasingly recognized.

Humanized and chimeric mAb can be administered to block critical cancer signaling pathways, induce tumor cell apoptosis or promote antibody-dependent killing of cancer cells. In addition to antibodies that target tumor antigens, antibodies that target the tumor microenvironment slow tumor growth either by enhancing host immune responses to TAA or by curtailing pro-tumorigenic factors produced in the tumor stroma. These attributes of target specificity, low toxicity and the ability to activate the immune system suggest the continuing promise of therapeutic antibodies.

Trastuzumab and Cetuximab are mAb approved for clinical use that target the human epidermal growth factor receptor 2 (HER-2) and the epidermal growth factor receptor (EGFR), critically required for the progression of some tumors [90]. Moreover, the mAb against CD20 molecule on the surface of B cells, Rituximab, is used in the treatment of B cell lymphomas [90]. Among their interference with cancer cell signaling, mAb promote tumor cell death through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxocity (CDC) [90]. Current studies are addressing the potential efficacy of mAb that target costimulatory molecules on immune cells such as OX40, member of the tumor necrosis factor receptor superfamily, and that antagonize inhibitory receptors such as the Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and the Programmed Death 1 (PD-1) [90]. Although these mAb can induce disease regression, a dose-dependent toxicity, in terms of autoimmune or autoinflammatory side effects, has been reported [91, 92]. Monoclonal antibodies may also be used in combination with exogenously administered cytokines, as IL-2, GM-CSF or G-CSF[90]. Among all, the effects of the combination of G-CSF with Rituximab have been studied in a Phase I/II clinical trial in patients with low grade lymphoma [93]. Instead of an overall response rate similar to that reported for Rituximab alone, the period of remission was remarkably longer.

Of utmost interest, the next generation of mAb working as carriers to provide greater specificity for cytotoxic agents such as radioisotopes and toxins and of unconjugated antibody therapies will undoubtedly yield many effective

new treatments for cancer over the next decade. These advances will arise from the identification and validation of new targets, the manipulation of tumor–host microenvironment interactions, and the optimization of antibody structure to promote the amplification of antitumor immune responses. One could easily envision combining mAb with a variety of specificities and antitumor mechanisms for patient-specific “cocktails” .

1.3.3 Adoptive Cell Transfer

Adoptive Cell Transfer (ACT) refers to the administration of huge numbers of tumor-specific T cells as anticancer therapy. These cells can be derived from the tumor environment (such as tumor-infiltrating lymphocytes (TIL)), from peripheral blood or they can be genetically modified to express a high affinity anti-tumor TCR.

ACT studies in experimental tumor models, dating back to the 1960’s, provided the earliest evidence that splenocytes from immunized animals sustain a therapeutic effect when used to treat transplanted syngeneic tumors [94, 95]. Subsequent studies have been aimed at defining the phenotype of the transferred T cells determining the ACT efficacy. Early experiments in mice showed that T-cell populations that had been cultured with leukemia cells could eradicate an established leukemia *in vivo* [96, 97], thus suggesting the involvement of memory cellular immunity. Strengthening this observation, following studies have demonstrated on one hand that the antitumor efficacy of ACT is related to the transfer of CD8 and CD4 T cells [98, 99], and on the other hand that T differentiation status deeply influences ACT outcome. In fact, adoptively transferred cells with a $CCR7^+$,$CD27^+$,$CD28^+$,$CD62L^+$ phenotype, proper of central memory T cells, were proved more effective than highly differentiated cells that lost these markers [100].

The clinical feasibility of this strategy was first shown in post-transplant lymphoproliferative disease, which is an Epstein-Barr virus (EBV)– associated B cell lymphoma that develops under conditions of severe T cell immunosuppression. Infusion of EBV-specific T cells led to the rejection of lymphomas and restored the severely hampered EBV-targeted immunity [101]. In 2002, the National Cancer Institute reported the outcome of a Phase II study of adoptive transfer of tumor-reactive TIL in pretreated patients with metastatic melanoma [22]. TIL obtained from a melanoma metastasis were grown *in vitro* with high dose IL-2 for overcoming the tumor-induced anergic state. Large amounts of cultured cells were re-infused into patients. Preconditioning the host with cyclophosphamide and fludarabine for inducing severe lymphocytopenia, was shown to be necessary for the survival and expansion of the infused TIL. Single-arm Phase II studies from separate institutions have shown objective response rates of 50% in patients with metastatic melanoma who are receiving this treatment [102, 103]. Approximately 10% of patients obtain a complete response, which may be durable. Side effects resulting from the infusion of TIL were mostly mild, consisting of vitiligo or uveitis in some patients, but both the non-myeloablative chemotherapy and high-dose IL-2 resulted in significant well-known toxicities.

As well as the cost and labor intensity, a major drawback of this approach is that TIL cannot be cultured from all patients. Moreover, the specificity of T cells within the TIL graft that are responsible for the clinical effects is unknown. This strategy could be refined by cloning high-affinity tumor-specific TCR from these cells that can serve as donor TCR to genetically modify unselected peripheral blood T lymphocytes for adoptive transfer [104]. This so-called TCR gene transfer is currently being tested in several clinical trials [105]. In order to use this strategy, the choice of target antigen is of utmost importance. Lethal toxicity can be induced by infusion of T lymphocytes genetically engineered to express TCR targeting antigens common to the tumor and the host [106, 107]. By contrast, antigens that have their expression restricted to tumor cells, such as cancer/testis antigen 1 (NY-ESO-1) in melanoma patients or CD19 in non-Hodgkin’s lymphoma B cell patients [108], can be targeted by TCR-redirected T cells thus reducing the risk of inducing systemic toxicity.

Along with ACT progress it remains to be seen whether the morbidity associated with any resultant autoimmune disease outweighs the antitumor benefit.

1.3.4 Cytokines

Cytokines are proteins secreted by cells that affect the immune response, typically via effects on other cells through receptors. Anticancer behavior of cytokines is generally believed to be mediated by their action on immune cells [109]. It is not surprising that numerous cytokines have attracted interest in the context of cancer therapy, including hematopoietic colony-stimulating factors, interferons, at least 35 different interleukins, tumor-necrosis factor, and a number of other protein ligands (reported in *Lewko, Principles of Cancer Bioth, 2009*).

There are a number of cytokines that have received FDA approval, including erythropoietin (EPO), G-CSF, and GM-CSF. At this immunotherapeutic crossroad, the two cytokines specifically approved as cancer therapy are type I IFN and IL-2.

1.3.4.1 *Type I IFN*

Type I IFN emerge as exquisite candidates for anti-cancer therapy. The power of this cytokine family in the scenario of neoplasias is mainly related to their antiangiogenic and proapoptotic effects on the tumor side and the positive conditioning of innate and adaptive immunity on the host side.

Type I IFN are a large family of immune regulatory proteins consisting of 14 functional IFNα cytokines, and one IFNβ cytokine sharing the same receptor and exerting similar biological activities. The ligand binding to the heterodimeric receptor (IFNAR-1 and IFNAR-2) [110, 111] complex leads to the tyrosine phosphorilation and activation of IFNAR-1-associated Tyk2 and IFNAR-2-associated Jak1, which, in turn, phosphorilate cytosolic Stat1 and Stat2, giving rise to two types of activated transcription factors: a Stat1 homodimer and IFN-stimulated gene factor 3 (ISGF3), a tripartite complex of phosphorilated Stat1, Stat2, and the constitutive DNA binding protein IFN regulatory factor-9 (IRF-9). The activated transcription factors enter the nucleus, bind to specific enhancer elements sequences on certain genes, and by regulating the expression of these genes, induce the wide range of IFN-dependent effects on cellular function [112-115]. Comparative functional analyses of several members of the type I IFN family revealed that they induce a significantly overlapping array of biologic responses, although quantitative differences have been noted in the specific activities of each particular form [116, 117]. A small number of genes have been identified that are differentially regulated by IFNα *vs* IFNβ [118, 119].

Type I IFN were discovered in 1957 by Isaacs and Lindenmann as soluble proteins able to inhibit virus replication in cell cultures [120-122]. More than 50 years of research revealed a panoply of antitumor biologic effects. Indeed, type I IFN have a long record use in clinical oncology. Even though today some new anticancer drugs have somehow replaced such cytokines in the treatment of certain hematological malignancies (hairy cell leukemia and chronic myeloid leukemia), type I IFN are still widely used for the treatment of metastatic melanoma, renal cell carcinoma and Kaposi sarcoma [123, 124].

For a long time, it was thought that the direct inhibitory effects on tumor cell growth/functions were the major mechanisms involved in the antitumor response in IFN-treated patients. In fact, type I IFN can directly inhibit the proliferation of normal and tumor cells *in vitro* and *in vivo*, and can exert other direct effects on tumor cells, including downregulation of oncogene expression, induction of tumor-suppressor genes, and increase of major histocompatibility complex (MHC) class I expression [125].

In addition to the direct effects on tumor cells, type I IFN exert several effects on host immune cells that can play a pivotal role in the overall antitumor response [126]. In the early 1990s, Ferrantini and colleagues carried out an ensemble of studies characterizing the effect of local production of type I IFN at the tumor site and discovering their ability to switch a highly tumorigenic behavior into an immunogenic one. Highly metastatic Friend leukemia cells (FLC) genetically modified to secrete IFNα1 exhibited a marked loss of their tumorigenic potential when injected into syngeneic mice. Likewise, these genetically modified IFN-producing tumor cells inhibited the growth of metastatic parental cells in transplantation assays [127]. The findings that IFNα1-FLC immune rejection and subsequent immune protection against a rechallenge was mostly mediated by CD8 T cells [128], implied that memory T cells were generated upon exposure of mice to the IFNα1-producing cells. In fact, a marked proliferation of CD8 T lymphocytes, especially among memory-phenotype $CD44^{hi}$ cells, in both the spleen and the lymph nodes, was observed after injection of viable tumor cells producing IFNα. Tumor cell-targeted cytokine gene therapy has been widely evaluated in animal models by many groups with different approaches, comprising both the use of genetically modified cells and the *in vivo* delivery of IFNα genes via injection of viral vectors or plasmid DNA [129].

Today, new attention is given to type I IFN as important factors bridging innate and adaptive immunity. Along with the understanding of the cytokine network regulating Th cell functions, several studies provided evidence on the importance of type I IFN in the differentiation of the Th1 subset, as well as in the generation and activity of CTL [124]. In particular, type I IFN are important for the *in vivo* expansion and long-term survival of CD8 T cells in response to specific antigens [130] and for the adjuvant activity on T cells induced by CpG DNA administration [131]. Type I IFN also prolong the survival of T cells in mice and the expression of anti-apoptotic genes in human primary T lymphocytes [132]. Similarly, a new interest in type I IFN as a "bridge system" linking innate and adaptive immunity stemmed from the identification of "natural IFN-producing cells" (the rare blood cell population that produces 200-1000 times more IFN than other blood cells after microbial challenge) also defined as plasmacytoid DC [133]. Type I IFN also affect monocyte and/or macrophage function and differentiation. Thus, these cytokines markedly support the differentiation of monocytes into partially mature DC with high capacity for Ag presentation [145], stimulate macrophage antibody-dependent cytotoxicity, and positively or negatively regulate the production of various cytokines (e.g., TNF, IL-1, IL-6, IL-8, IL-12, and IL-18) by macrophages [146]. In addition, treatment of DC with IFN-α/β has been shown to upregulate surface expression of MHC class I, class II, and costimulatory molecules both in animal models and in the human system (43–45), and to augment the capacity of DC to stimulate CD4 and CD8 T cell responses. Interestingly, it has been observed that injection of type I IFN stimulates efficient cross-priming of antigen-specific CD8 T lymphocytes *in vivo* through a direct involvement of DC [147].

It has recently been reported that type I IFN can greatly enhance cross-presentation and CD8 T cell cross-priming by stimulating CD8α DC that have engulfed tumor cells undergoing immunogenic apoptosis by CTX treatment

[37, 133]. A deep mechanistic study revealed that type I IFN favor cross-priming through multiple actions on CD8α DC, i.e., (i) by promoting intracellular Ag persistence through phagosomal alkalinization and, thus cross-presentation; (ii) by sustaining the survival of Ag bearing DC selectively through the upmodulation of antiapoptotic genes; and (iii) by activating DC. Overall, these data suggest that type I IFN cross-prime CD8 T cells against apoptotic cell-derived Ag both by licensing DC and by enhancing cross-presentation [37, 134]. Recently, Schreiber and collaborators revealed an obligate role for type I IFN in cancer immunoediting thus strengthening the definition of these cytokines as central coordinators at the tumor-host interface [135, 136]. Endogenously produced type I IFN are required, in immunocompetent mice, for rejection of highly immunogenic 3'-methylcholanthrene-induced (MCA) sarcomas and also prevent the outgrowth of primary carcinogen-induced tumors. Furthermore, several MCA sarcomas derived from IFNAR1-/- mice were rejected in a lymphocyte-dependent manner in wild-type mice, thus suggesting that tumors arising in the absence of IFN-αβ responsiveness are more immunogenic as a group than tumors arising in immunocompetent mice that almost invariably form progressively growing tumors when transplanted into wild-type recipient. A more recent study from same group, elucidated the role of endogenous type I IFN in driving host-protective, antitumor responses. They showed that type I IFN act early during the initiation of the immune response and that innate immune cells represent the essential responsive cells for the generation of protective antitumor immunity. Whereas type I IFN-unresponsive mice showed a defect in the priming of tumor-specific CTL, reconstitution of type I IFN sensitivity in innate immune cells was sufficient to restore this deficit and resulted in tumor rejection. Type I IFN major physiological function is selectively directed toward a single host cell population i.e., DC, and, at least in part, type I IFN function to enhance the capacity of $CD8\alpha^+$ DC to cross-present antigen to CD8 T cells, thus playing an essential role in tumor-specific T cell priming and tumor elimination.

Type I IFN, and in particular IFNα, became the first biological or immune therapy approved as an anticancer treatment when it received regulatory approval in 1986. There were two recombinant DNA products that were extensively studied and eventually approved for widespread use, IFNα2a (Intron A®) and IFNα2b (Roferon®).

Lessons taken from the history of IFN research have allowed to define and ameliorate the modalities of clinical use of these immune response modifiers. In a recent study published by Kirkwood and colleagues in melanoma patients treated with the high-dose IFNα regimen [137], a striking correlation between clinical response to IFNα and autoimmune events was observed. The results of this study, strongly supporting the concept of IFNα acting as an immune adjuvant, are somehow consistent with the hypothesis of a possible role of DC in the pathogenesis of autoimmune responses [138] and may lead to new perspectives for identifying categories of patients responding to the IFNα therapy [139].Similarly, this study further supports the interest of using IFNα in association with cancer vaccines. In this regard, a pilot Phase I-II trial to determine the effects of IFNα, administered as an adjuvant of Melan-A/MART-1:26–35(27L) and gp100:209–217(210M) peptides in stage IV melanoma patients has recently been carried out [140], providing a first experimental rationale in humans for the use of these cytokines as an adjuvant of cancer vaccines.

On the whole the "state-of-the-art" role of type I IFN in tumor immunity and immunotherapy offers new opportunities for fostering interactions between clinicians and researchers with the common goal of achieving a rapid clinical exploitation of the emerging knowledge in the field.

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Cancer Research

Cyclophosphamide Synergizes with Type I Interferons through Systemic Dendritic Cell Reactivation and Induction of Immunogenic Tumor Apoptosis

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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; 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 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 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 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, 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-II$^-$CD11c$^+$B220$^+$, Lin$^-$MHC-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-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 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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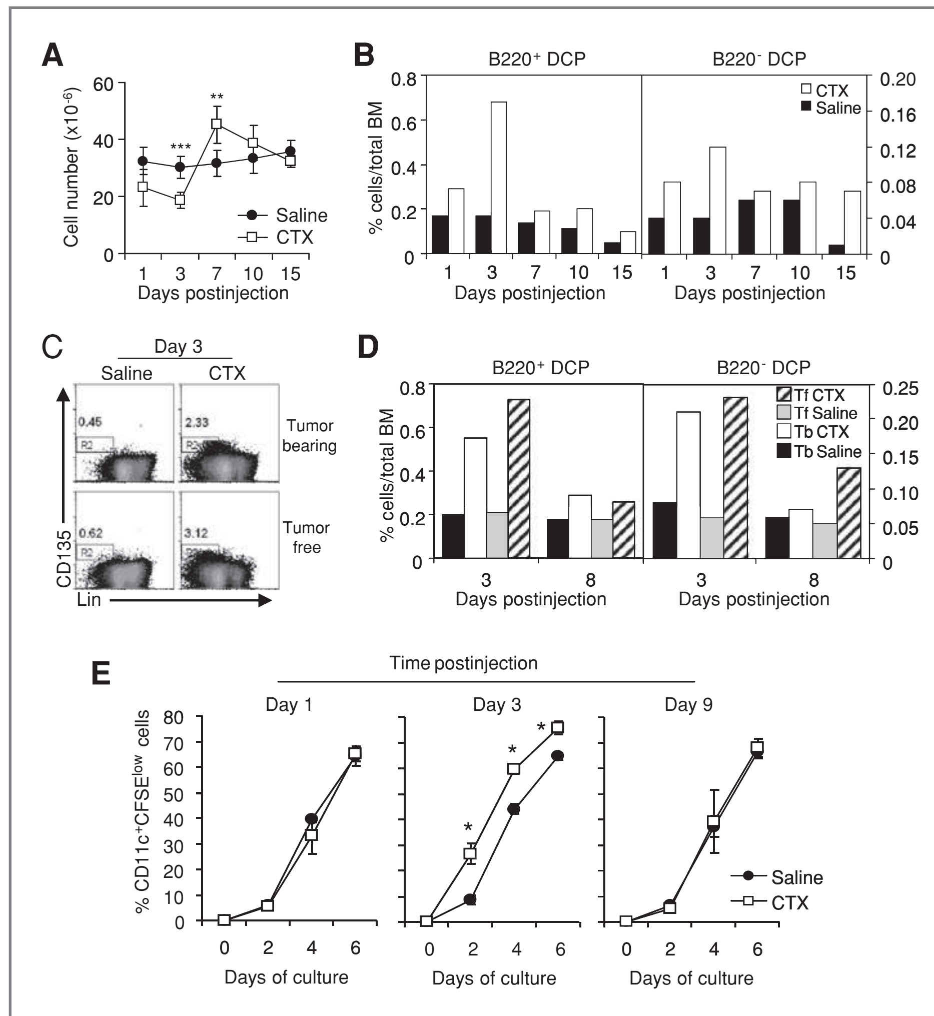

Figure 1. Effect of CTX injection on BM mobilization and DC homeostasis. EG7 tumor-bearing mice were injected i.p. with CTX or saline. At the indicated time points, BM was extracted. A, total BM cell counts in each individual mouse (mean ± SD). B, relative frequency of $B220^+$ and $B220^-$ DCPs in whole 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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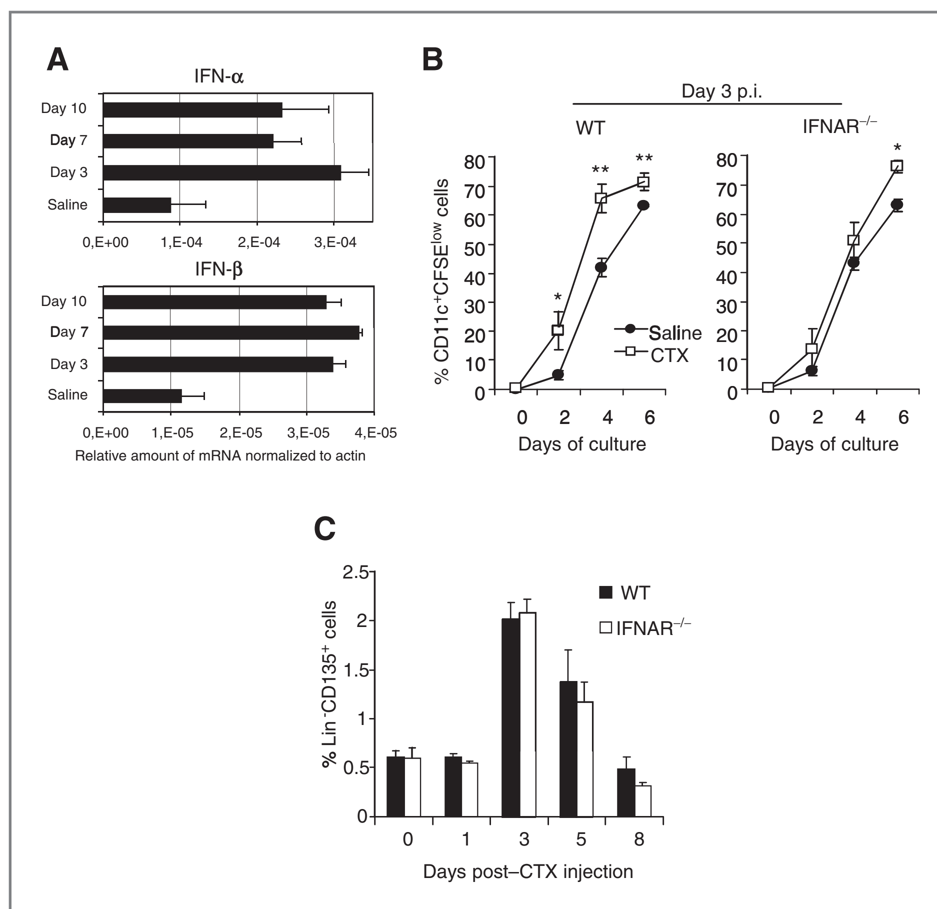

Figure 2. Role of IFN-I in CTX-induced DC mobilization. A, quantitative reverse transcriptase PCR (qRT-PCR) of BM at various time points post–CTX treatment. Data represent the relative amount of IFN-α and IFN-β mRNA normalized to β-actin (mean ± SD). One of 3 representative experiments is shown. B, GM-CSF 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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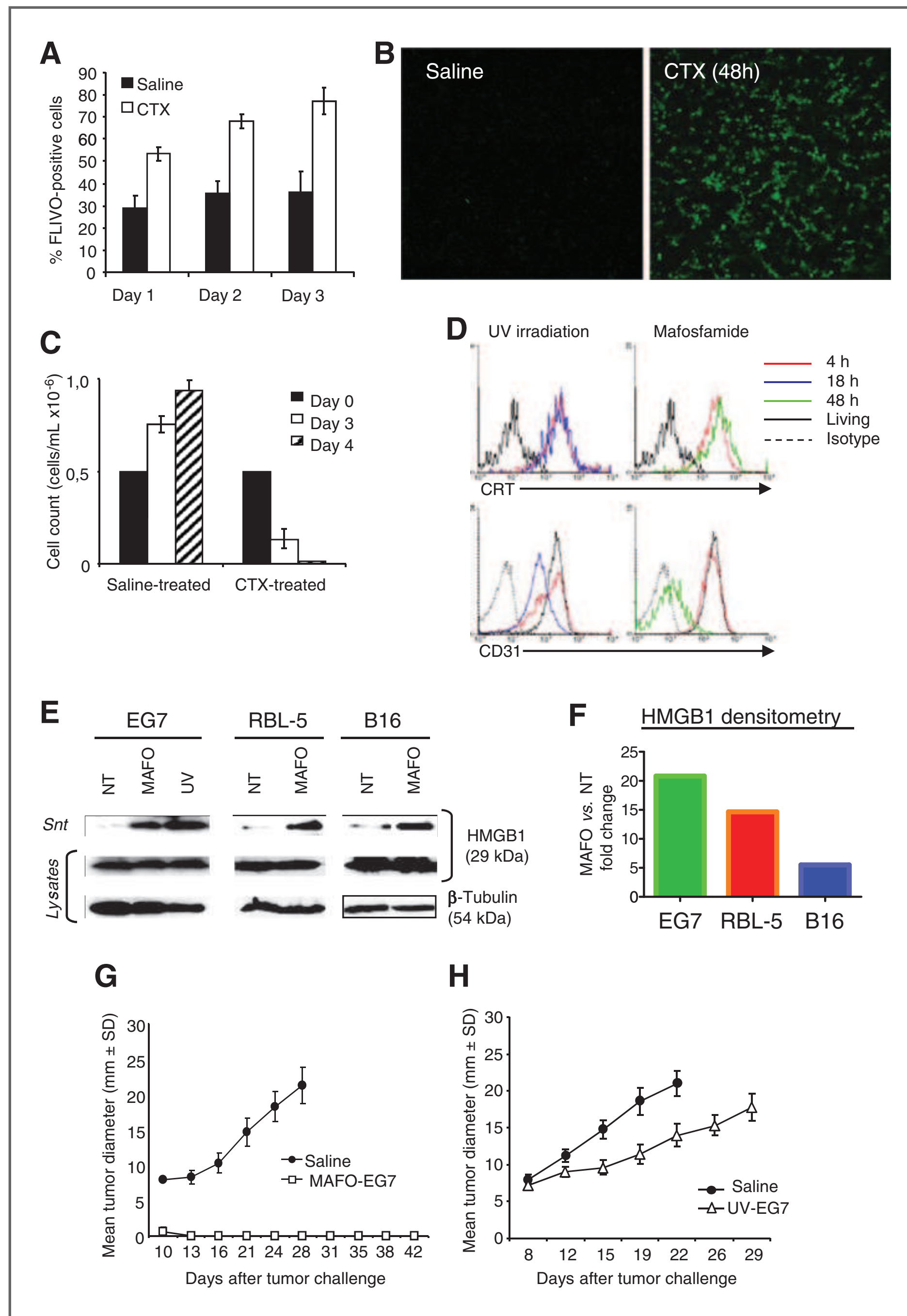

Figure 3. Induction of immunogenic apoptosis by CTX. Tumor-bearing mice were treated with FLIVO at the indicated times p.i. A, FACS of FLIVO staining in tumor cell explants. B, CLSM of tumor sections. Bars, 100 µm. C, cell counts of *ex vivo* cultured tumor explants from mice at day 3 p.i. One of 3 experiments 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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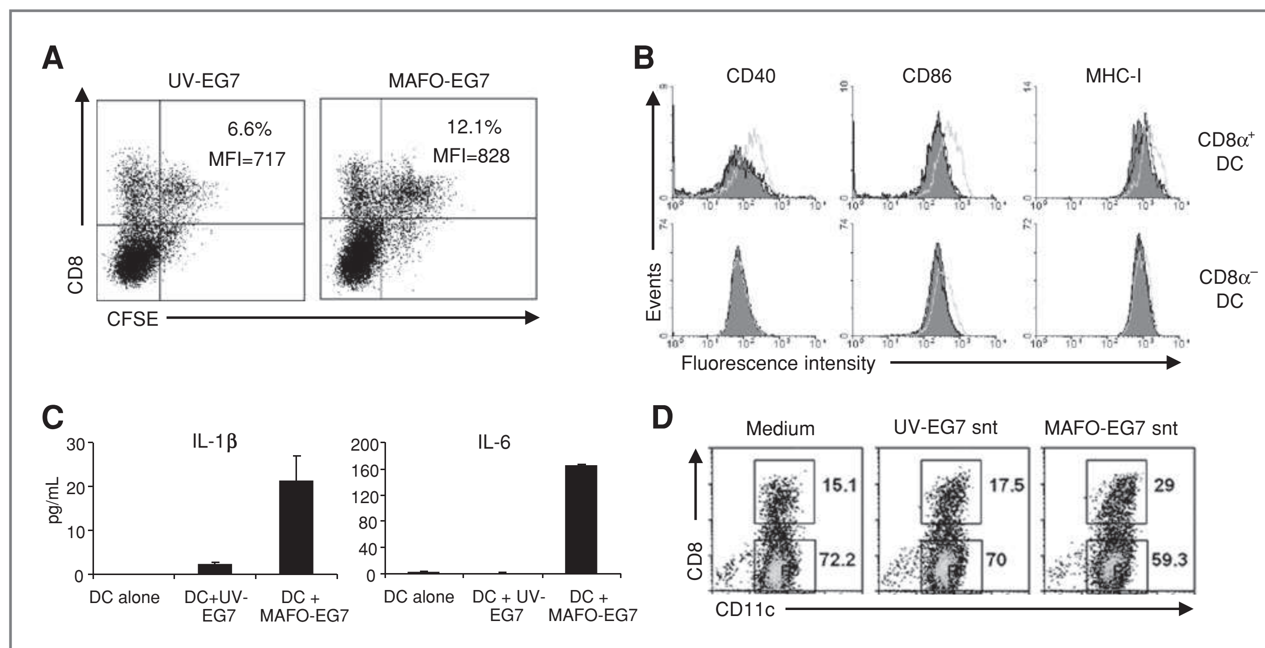

Figure 4. MAFO-EG7 cell uptake by DC. A, naive DC were cocultured with CFSE-labeled MAFO-EG7 or UV-EG7 cells. Uptake by CD8α$^+$ DC was measured 18 hours later by FACS as CFSE$^+$. B, phenotype of DC subsets after 18-hour culture with snts from MAFO-EG7 (gray opened), UV-EG7 cells (black opened), or 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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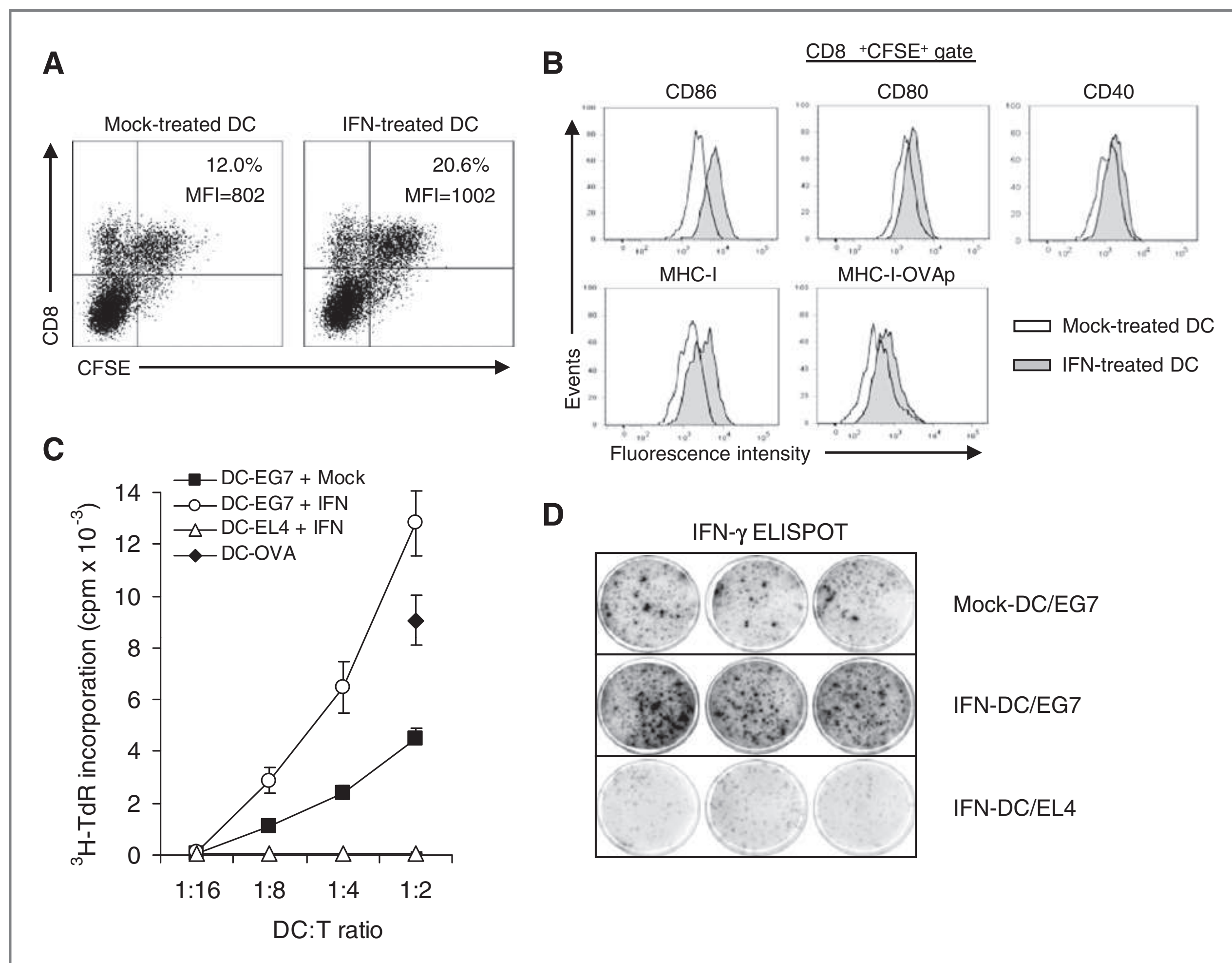

Figure 5. Effect of IFN-I on cross-presentation of EG7-derived OVA by DC. A, uptake by CD8α$^+$ DC of CFSE$^+$ MAFO-EG7 cells after 18-hour culture with IFN-I or mock. B, phenotype of IFN-treated or mock-treated CD8α$^+$ CFSE$^+$ DC. One of 3 experiments is shown. C, proliferative response of OT-1 CD8 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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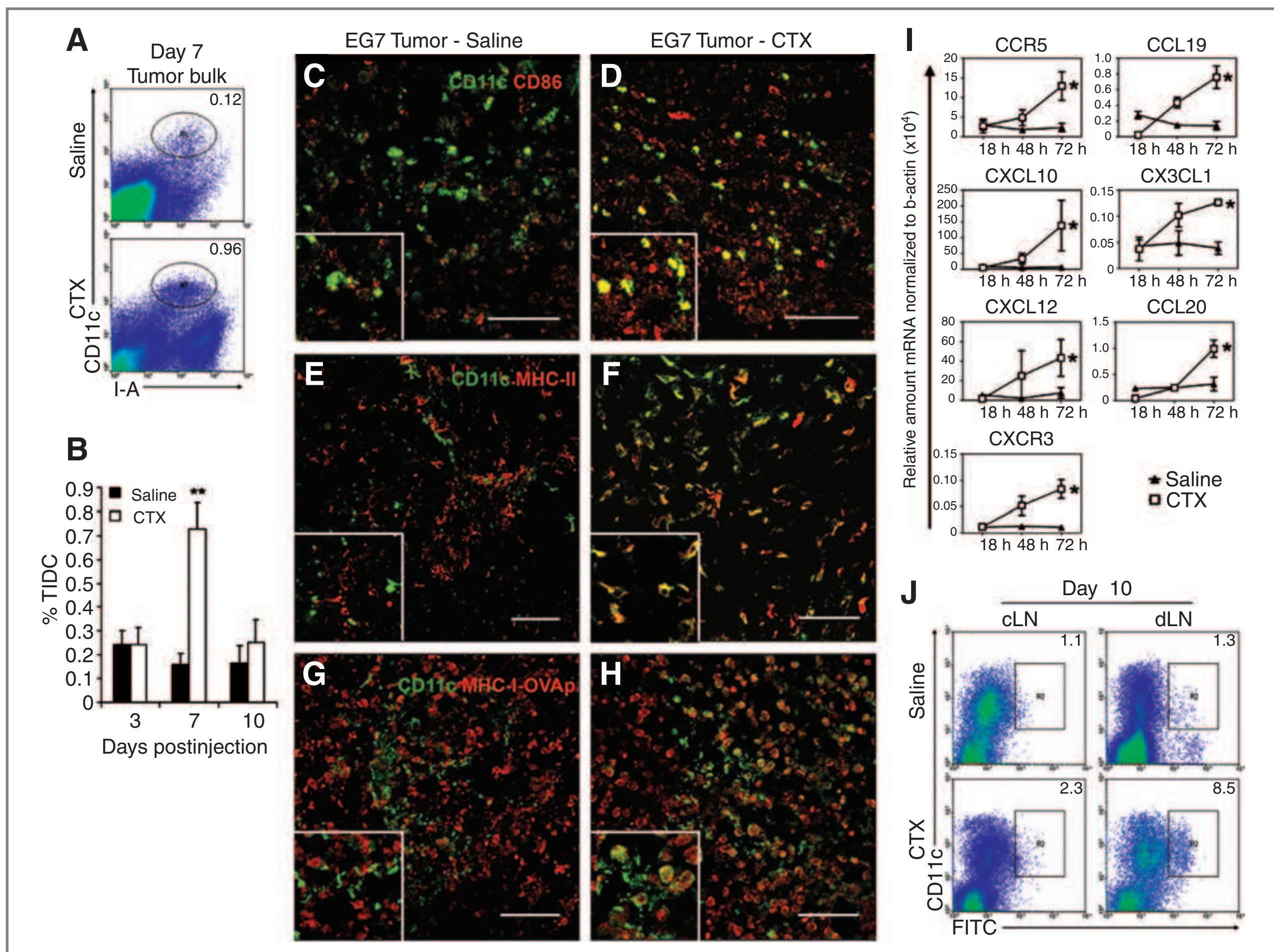

Figure 6. Tumor infiltration and LN homing of DC after CTX. A, CD11c$^+$I-A$^+$ TIDC at day 7 p.i. (CD3$^-$CD19$^-$ gate) in tumor bulk. B, kinetic analysis of TIDC in tumor explants at various times p.i. Histograms represent mean frequencies ± SD of 1 of 3 individual mice. **, $P < 0.01$. One of 4 experiments 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 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 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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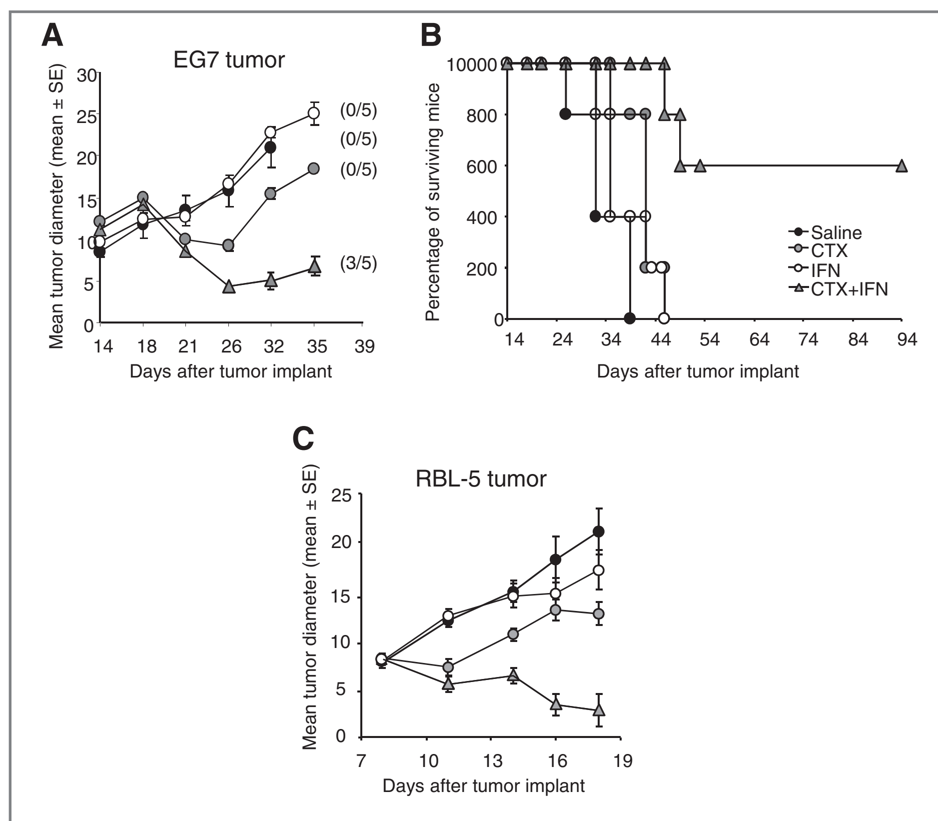

Figure 7. Antitumor effect of combined CTX/IFN-I treatments. Mice bearing implanted tumors were injected i.p. with CTX, followed by 4 peritumoral injections of IFN-I. A, EG7 tumor growth expressed as mean diameter ± SE (5 mice/group). Number of surviving mice is indicated in brackets. B, mortality over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

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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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Type I IFNs Control Antigen Retention and Survival of CD8α+ Dendritic Cells after Uptake of Tumor Apoptotic Cells Leading to Cross-Priming

Silvia Lorenzi,[1] Fabrizio Mattei, Antonella Sistigu, Laura Bracci, Francesca Spadaro, Massimo Sanchez, Massimo Spada, Filippo Belardelli, Lucia Gabriele, and Giovanna Schiavoni

Cross-presentation is a crucial mechanism for generating CD8 T cell responses against exogenous Ags, such as dead cell-derived Ag, and is mainly fulfilled by CD8α+ dendritic cells (DC). Apoptotic cell death occurring in steady-state conditions is largely tolerogenic, thus hampering the onset of effector CD8 T cell responses. Type I IFNs (IFN-I) have been shown to promote cross-priming of CD8 T cells against soluble or viral Ags, partly through stimulation of DC. By using UV-irradiated OVA-expressing mouse EG7 thymoma cells, we show that IFN-I promote intracellular Ag persistence in CD8α+ DC that have engulfed apoptotic EG7 cells, regulating intracellular pH, thus enhancing cross-presentation of apoptotic EG7-derived OVA Ag by CD8α+ DC. Notably, IFN-I also sustain the survival of Ag-bearing CD8α+ DC by selective upmodulation of antiapoptotic genes and stimulate the activation of cross-presenting DC. The ensemble of these effects results in the induction of CD8 T cell effector response in vitro and in vivo. Overall, our data indicate that IFN-I cross-prime CD8 T cells against apoptotic cell-derived Ag both by licensing DC and by enhancing cross-presentation. ***The Journal of Immunology*, 2011, 186: 5142–5150.**

Cross-presentation of cell-associated Ag, such as dead cell-derived Ag, is a crucial process for generating CD8 T cell responses to Ag that are not expressed by APC, such as viruses that do not infect APC or tumors of nonhematopoietic origin (1). Among APC, dendritic cells (DC) are specialized for cross-presentation, and accumulating literature indicates that in vivo this process is mainly fulfilled by the CD8α+ DC subset (2). In the steady state, CD8α+ DC constitutively cross-present self Ag, such as material derived from apoptotic cells as a result of constitutive cell turnover, leading to self-tolerance (3). However, in the context of infection or pathological distress, signals consisting of microbial compounds or of inflammatory stimuli released by cells of innate immunity act as danger signals that induce DC activation, in a process referred to as licensing of DC to cross-priming (4). The underlying molecular mechanisms that result from such licensing are still under investigation and include enhanced costimulatory signals and diminished proapoptotic signals by the DC (5). It has been reported that triggering of TLR4 increases the efficiency of peptide presentation on MHC-I molecules by DC, suggesting that the enhancement of cross-presentation may represent another mechanism promoting cross-priming (6). With regard to the mechanisms regulating cross-presentation, it is known that presentation of particulate Ag, such as cellular Ag, is critically dependent on the timing of persistence within phagosomal compartment, a process governed by intraphagosomal pH (6). Studies from Amigorena laboratory have established a strict correlation between phagosomal alkalinization, which delays the proteolytic activity of lysosomal enzymes, and the efficiency of cross-presentation (7).

Type I IFNs (IFN-I) are a family of inflammatory cytokines produced by innate cells upon pathogenic challenge, playing multiple roles in the stimulation of immune responses, including DC activation and CD8 T cell effector function in vitro and in vivo. Of interest, IFN-I have been shown to promote cross-priming against viral or protein Ag, partly through the stimulation of DC (8–10). Furthermore, cross-priming stimulated by TLR3, TLR4, and TLR9 ligands was shown to be critically dependent on IFN-I signaling, implying these cytokines as important mediators in infection-stimulated cross-priming (9). Recent work from our laboratory has shown that human DC generated in the presence of IFN-α exhibit enhanced cross-presentation of allogeneic apoptotic cell Ag to autologous CD8 T cells, suggesting a role for IFN-I in DC cross-presentation of cell-associated Ag (11). In this study, we demonstrate that IFN-I promote cross-priming in vivo against cell-associated Ag derived from tumor apoptotic cells through multiple actions on CD8α+ DC, as follows: 1) by enhancing Ag persistence and, thus, cross-presentation; 2) by sustaining the survival of Ag-bearing DC selectively; and 3) by activating DC.

Materials and Methods

Mice

Female C57BL/6 mice (5–7 wk old) and OT-I TCR-transgenic mice were purchased from Charles River Laboratories. IFN regulatory factor (IRF)-

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The online version of this article contains supplemental material.

Abbreviations used in this article: apoEG7, apoptotic EG7; CLSM, confocal laser-scanning microscopy; C_T, threshold cycle; DC, dendritic cells; DPI, diphenylene iodonium; IFN-DC, IFN-treated DCs; IRF, IFN regulatory factor; LN, lymph node; MFI, mean fluorescence intensity; MHC-OVAp, MHC class I molecule K^b bound to the peptide SIINFEKL of OVA; NOX2, NADPH oxidase 2; NT-DC, untreated DC; PI, propidium iodide; WT, wild-type.

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$8^{-/-}$ mice were generated and bred, as described (12). All mice were manipulated in accordance with the local Ethical Committee guidelines.

Reagents

High-titer IFN-I was prepared from the C243-3 cell line, as described in detail elsewhere (13). Anti-mouse IFN-I sheep Ig was used at 1000 neutralizing units (14). For flow cytometry, the following mAbs were used: anti-CD11c, which was used either in FITC, PE, or allophycocyanin form; PE anti-CD8, CD25, and CD69; biotin anti-CD40, CD86, and I-A (all from BD Pharmingen); tricolor anti-CD8 (Caltag Laboratories); and biotin anti-mouse MHC class I molecule K^b bound to the peptide SIINFEKL of OVA (MHC-OVAp; clone 25-D1.16; eBioscience). Biotinylated mAbs were detected with streptavidin PerCP (BD Pharmingen) or streptavidin tricolor (Caltag Laboratories). Lysosensor green DND-189 (Invitrogen) was used at 5 μM. Diphenylene iodonium (DPI; Sigma-Aldrich) was used at 5 μM.

Isolation of DC and OT-I lymphocytes

The procedure of splenic DC isolation has been described in detail elsewhere (15). Briefly, total splenocytes were subjected to density-gradient centrifugation in Nycodenz solution (1077 g/ml; Life Technologies). The low-density fraction was magnetically sorted using anti-CD11c microbeads (Miltenyi Biotec), yielding routinely >95% $CD11c^+$ cells. The DC purified by this method are virtually free from T cell contaminants (<0.1% $CD3^+$ cells; data not shown). In some experiments, $CD11c^+$ cells were further sorted into $CD8\alpha^+$ and $CD8\alpha^-$ subsets using FACSAria cell sorter (BD Biosciences), yielding 99% purity. CD8 T cells from OT-I splenocytes and lymph node (LN) cells were purified using anti-CD8 microbeads (Miltenyi Biotec). Purity routinely ranged ~95–98%.

Culture and apoptosis induction of EG.7-OVA cell line

The EG.7-OVA cell line (EG7, CRL-2113; American Type Culture Collection), a stable transfectant of the murine OVA-expressing EL4 thymoma ($H-2^b$), was maintained in complete RPMI 1640 medium supplemented with G418 (0.4 mg/ml; Calbiochem). For uptake assays, EG7 cells were labeled with the dye CFSE (1 μM; Invitrogen). Cells were then washed and resuspended in PBS/1% FCS at 6×10^6/ml and UV irradiated (λ= 254 nm) at a 9-cm distance for 3 min. Cells were then resuspended in IMDM complete medium (4×10^6 cells/ml) and incubated at 37°C, 5% CO_2 overnight. Apoptosis was FACS analyzed by annexin V (Roche) and propidium iodide (PI; Sigma-Aldrich) staining (data not shown).

Assays for apoptotic cell uptake

Splenic DC were cocultured with apoptotic EG7 (apoEG7) cells at a 1:4 ratio in the presence or absence of IFN-I (5×10^3 U/ml) for 3–18 h at 37°C, 5% CO_2. To assess uptake in vitro, DC were first separated from excess of apoEG7 cells not taken up by the DC by Nycodenz density-gradient centrifugation (a method allowing an enrichment of the DC fraction from 20% to 75–85%), and then surface stained with a panel of mAbs. The presence of apoptotic bodies ($CFSE^+$) in $CD11c^+CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DC subsets and their phenotype was analyzed by FACS. For confocal laser-scanning microscopy (CLSM), DC were left to adhere on poly(lysine)-treated glass slides (Menzel Glasser) for 20 min at 4°C. Cells were stained with biotin anti–I-A for 30 min at 4°C, followed by streptavidin-Alexa 594 (Molecular Probes), washed, and fixed in 3% paraformaldehyde for 30 min at 4°C. Cover glasses were then mounted on microscope slides with antifade Vectashield reagent (Vector Laboratories). CLSM observations were performed using a Leica TCS SP2 AOBS apparatus. Signals from different fluorescent probes were taken in sequential scan mode. For in vivo uptake, CFSE-labeled apoEG7 cells (5×10^6 in 0.2 ml) were injected i.v. with or without IFN-I (10^5 U/mouse). Three to 18 h later, mice were sacrificed and splenic DC were analyzed by FACS for CFSE positivity. Quantitative analysis of CFSE fluorescence intensity in phagocytic cells was performed using ImageJ software (http://rsbweb.nih.gov/ij/).

OT-I T cell priming

For in vitro priming, OT-I CD8 T cells were labeled with CFSE (1 μM) and then seeded in 96-well U-bottomed plates (10^5 cells/well) together with decreasing numbers of DC previously loaded with apoEG7 cells. Cocultures were performed in triplicate and incubated at 37°C, 5% CO_2 for 3 d. Cells were then harvested and stained with anti-CD8, CD25, and CD69 mAbs and analyzed by FACS. CFSE dilutions and phenotypic activation in CD8 T cells were determined. For in vivo priming, naive C57BL/6 mice were injected i.v. with 2-5 $\times 10^6$ OT-I CD8 T cells previously labeled with CFSE (5 μM). The following day, recipients were immunized in the hind footpads with 5×10^5 apoEG7-DC alone or containing IFN-I (2.5×10^4 U/mouse). Three days later, the popliteal LN excised and the resulting cell suspensions were stained with anti-CD8, CD25, and CD69 mAbs and analyzed by FACS. In vivo priming was also assessed by injecting i.v. 20×10^6 apoEG7 cells alone or plus IFN-I (10^6 U/mouse) into IRF-$8^{-/-}$ or control recipients, adoptively transferred with CFSE-labeled OT-I cells. OT-I proliferative response was measured 3 d later in the spleen. Ag-specific IFN-γ production by CD8 T cells was assessed by ELISPOT assay using reagents and methods, as recommended by the manufacturer (Mabtech AB). IFN-γ spot-forming cells were analyzed by ImageJ software.

Quantitative RT-PCR

Quantitative RT-PCR in sorted populations of splenic DC was performed using Sensimix Plus SYBR kit containing the fluorescent dye SYBR Green (Quantace). Forward and reverse primers (Supplemental Table I) were purchased from Primm. Quality and specificity of amplicons in each sample were detected by dissociation curve analysis. Triplicates were performed for each experimental point. For quantization, threshold cycle (C_T) values were determined by the Sequence Detection System software (Applied Biosystems), and ΔC_T was obtained by subtracting C_T of reference gene, β-actin, from C_T of target gene. Gene expression was presented as relative amount of mRNA normalized to β-actin and was calculated as $2^{-\Delta Ct}$ (16).

Statistical analysis

Levels of significance for comparison between samples were determined by the two-tailed Student *t* test. The *p* values <0.05 were considered statistically significant.

Results

Apoptotic EG7 cells are selectively captured by $CD8\alpha^+$ DC in vitro and in vivo

To evaluate the effects of IFN-I on cross-presentation of Ag derived from apoptotic cells, we used OVA-expressing EG7 thymoma cells induced to apoptosis by UV irradiation. In mice, $CD8\alpha^+$ DC are the specialized population for cross-presentation, although such feature may not be attributed to an exclusive ability of these cells to capture exogenous Ag (17). However, accumulating evidence suggests that apoptotic cells are captured preferentially by $CD8\alpha^+$ DC (18, 19). We initially evaluated which DC subsets were able to capture apoEG7 cells. To this end, we cocultured magnetically sorted $CD11c^+$ splenic DC with apoEG7 cells for 3 h, then removed the excess of uneaten apoptotic fragments by Nycodenz density-gradient centrifugation, to enrich the DC fraction (80%; Fig. 1*A*), and analyzed the uptake in the two DC subsets by FACS. As expected, $CD8\alpha^+$, but not $CD8\alpha^-$, DC efficiently captured apoEG7 cells in vitro (Fig. 1*A*) and in vivo (Fig. 1*B*). To confirm the exclusive ability of $CD8\alpha^+$ DC to phagocytose apoEG7 cells, we repeated the experiment with DC from IRF-8–deficient (IRF-$8^{-/-}$) mice, characterized by reduced numbers and severe functional impairment of $CD8\alpha^+$ DC (20). In this setting, the only subset functionally competent for uptake is the $CD8\alpha^-$ DC. Notably, $CD8\alpha^-$ DC could not compensate for the absence of functionally competent $CD8\alpha^+$ DC, because no phagocytic activity could be observed in $CD8\alpha^-$ DC from IRF-$8^{-/-}$ mice, indicating that apoEG7 cells could be captured exclusively by $CD8\alpha^+$ DC (Fig. 1*C*).

IFN-I promote Ag retention by $CD8\alpha^+$ DC after uptake of apoEG7 cells

Next, we evaluated the effects of IFN-I on phagocytosis of apoEG7 by $CD8\alpha^+$ DC. To this end, we cocultured CFSE-labeled apoEG7 with splenic DC in the presence or absence of IFN-I for 3–18 h and measured the internalization of apoptotic bodies by DC. We found that although IFN-I treatment did not significantly affect the uptake of $CFSE^+$ apoEG7 cells by $CD8\alpha^+$ DC, as revealed by FACS at 3 h, it determined significant retention of phagocytosed material after 18 h (Fig. 2*A*). In fact, whereas at 3 h the percentages of $CD8\alpha^+CFSE^+$ were comparable in both cultures, at 18-h

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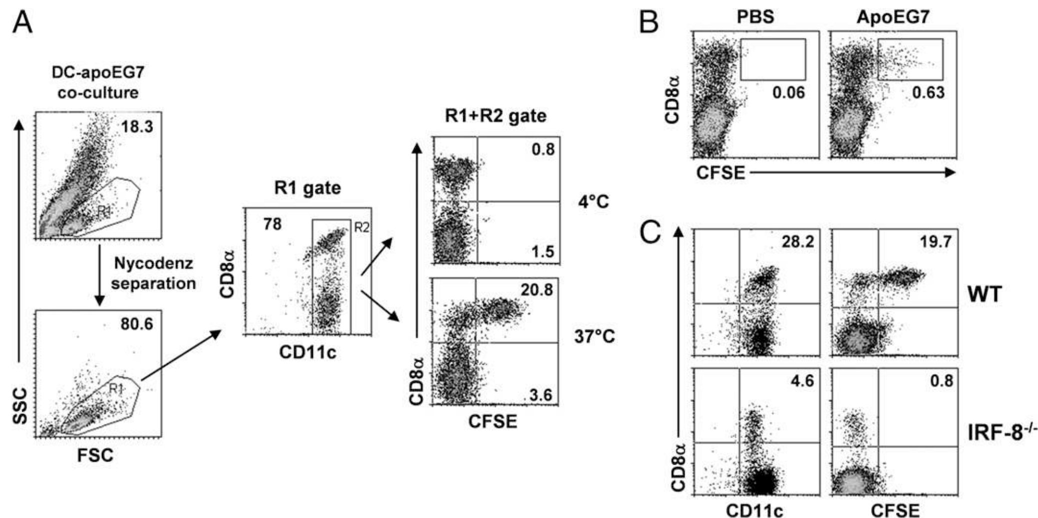

FIGURE 1. Selective uptake of apoEG7 cells by CD8α+ DC. *A*, Purified splenic CD11c+ DC were cocultured at a 1:4 ratio with CFSE-labeled apoEG7 cells for 3 h at 37°C or at 4°C, then separated from apoptotic cells by Nycodenz centrifugation, surface stained for CD8 and CD11c expression, and analyzed by FACS for uptake by gating on CD11c+ cells. Plots represent the percentage of CD11c+ DC engulfing apoEG7 cells (CFSE+). One representative experiment of nine is shown. *B*, Naive C57BL/6 mice were injected i.v. with 5 × 10^6 CFSE-labeled apoEG7 cells or PBS. Three hours later, splenic DC were magnetically sorted and uptake was evaluated by FACS analysis on gated CD11c+ cells. Representative data from four experiments are shown. *C*, Uptake by splenic CD11c+ DC from IRF-8$^{-/-}$ or WT C57BL/6 mice after 3-h coculture with CFSE-labeled apoEG7 cells at 37°C. One representative experiment of three is shown.

coculture 20.2% CD8α+CFSE+ were found in CD8α+ DC cultured in the presence of IFN-I with respect to only 8.3% CD8α+CFSE+ in control cells (Fig. 2*A*). Hence, in IFN-treated samples, the percentage of CFSE+CD8α+ cells found at 18 h was similar to that at 3 h (20.2 versus 21; Fig. 2*A*), whereas in untreated controls the amount of CFSE+CD8α+ cells dropped considerably at 18 h with respect to 3 h (8.3 versus 20.1; Fig. 2*A*). Analysis of mean fluorescence intensity (MFI) in CFSE+CD8α+ cells revealed that IFN-treated DC exhibited increased levels of green fluorescence with respect to untreated cells, suggesting a larger number of antigenic material carried (Fig. 2*B*). CLSM observations further evidenced a higher frequency of CFSE+ particles within the intracellular compartment of IFN-treated DC, with respect to untreated cells at 18 h (Fig. 2*C*). Densitometric analysis of CFSE fluorescence revealed a significant increase in the green fluorescence intensity retrieved in phagocytic IFN-treated DC, with respect to untreated DC (NT-DC), indicating a larger quantity of antigenic particles per cell at 18 h (Fig. 2*D*). This finding led us to hypothesize that IFN-I may control the persistence of apoptotic bodies within the phagosomal compartments of DC. Alternatively, although unlikely, IFN-I may prolong the endocytic activity of DC throughout the 18-h culture, meaning that the DC were continuously eating and processing the Ag. To test this, we cocultured DC with CFSE+ apoEG7 cells for 3 h, with or without IFN-I, then removed the excess of "uneaten" apoptotic bodies from the culture by density-gradient centrifugation and left the DC alone in the identical culture medium (containing or not IFN-I) for the remaining 15 h of culture (3 h + 15 h). In this setting, no more apoptotic bodies were available for DC to eat, thus meaning that the antigenic material to be retrieved within DC at 18 h would be the result of the unprocessed Ag only. Remarkably, the percentages of CD8α+ CFSE+ retrieved in samples of IFN-treated DC in which apoEG7 cells had been withdrawn at 3 h (3 h + 15 h) were similar to those found with DC undergoing continuous 18-h coculture (18 h), indicating that IFN-I effectively acted by prolonging Ag retention in CD8α+ DC (Fig. 2*E*). In contrast, untreated CD8α+ DC lost ~70% of antigenic cargo either in the continuous 18 h or in the 3 h + 15-h coculture setting with respect to 3-h cocultures (Fig. 2*E*).

To assess whether IFN-I could prolong Ag persistence within phagocytic CD8α+ DC in vivo, we injected CFSE-labeled apoEG7 cells in combination or not with IFN-I and analyzed the uptake by splenic CD8α+ DC after 3 and 18 h. Similarly to what was observed in vitro, we found that the uptake of apoptotic cells by CD8α+ DC in vivo was only marginally increased by IFN-I, as revealed by the percentage of phagocytosis found in IFN-I–treated and untreated DC at 3 h postinjection (0.34 versus 0.55%; Fig. 2*F*). Remarkably, CD8α+ DC from mice exposed to IFN-I treatment almost completely retained the antigenic cargo after 18 h (0.47%; Fig. 2*F*), whereas this was completely lost by cells of mice injected with apoEG7 alone (0.06%; Fig. 2*F*).

Role of intracellular pH alkalinization in IFN-induced prolonged Ag persistence

CD8α+ DC are thought to possess specialized machinery to direct endocytosed Ag into the MHC class I presentation pathway. In this regard, the Ag cross-presentation pathway is thought to be critically dependent on low proteolytic activity of lysosomal enzymes, a process requiring a high phagosomal pH. This physiological condition results in enhanced Ag storage within the intracellular compartments, allowing DC to display peptide within both MHC-I and MHC-II complexes (7). To address whether IFN-I treatment could modulate intracellular pH in phagocytic DC, at various times of coculture with apoEG7 cells we treated DC with Lysosensor green, a fluorescent acidotropic probe exhibiting a pH-dependent increase in MFI upon acidification. As shown in Fig. 3*A*, at 5–7 h postcoculture, DC treated with IFN-I exhibited a significant drop in the MFI, implying a more alkaline phagosomal pH with respect to untreated cells. To further assess the role of intraphagosomal pH in IFN-induced Ag retention by CD8α+ DC, we used DPI, an inhibitor of the activity of NADPH oxidase 2 (NOX2), a flavin-containing enzyme known to control pH alkalinization in CD8α+ DC phagosomes (21). DPI was added to IFN-treated and untreated DC-apoEG7 cocultures at 3 h, in order not to interfere with phagocytosis, and left until 18 h. As shown in Fig. 3*B*, addition of DPI significantly reduced the percentages of CFSE+CD8α+, but not of CFSE−CD8α+, cells in IFN-treated cultures, indicating a decrease

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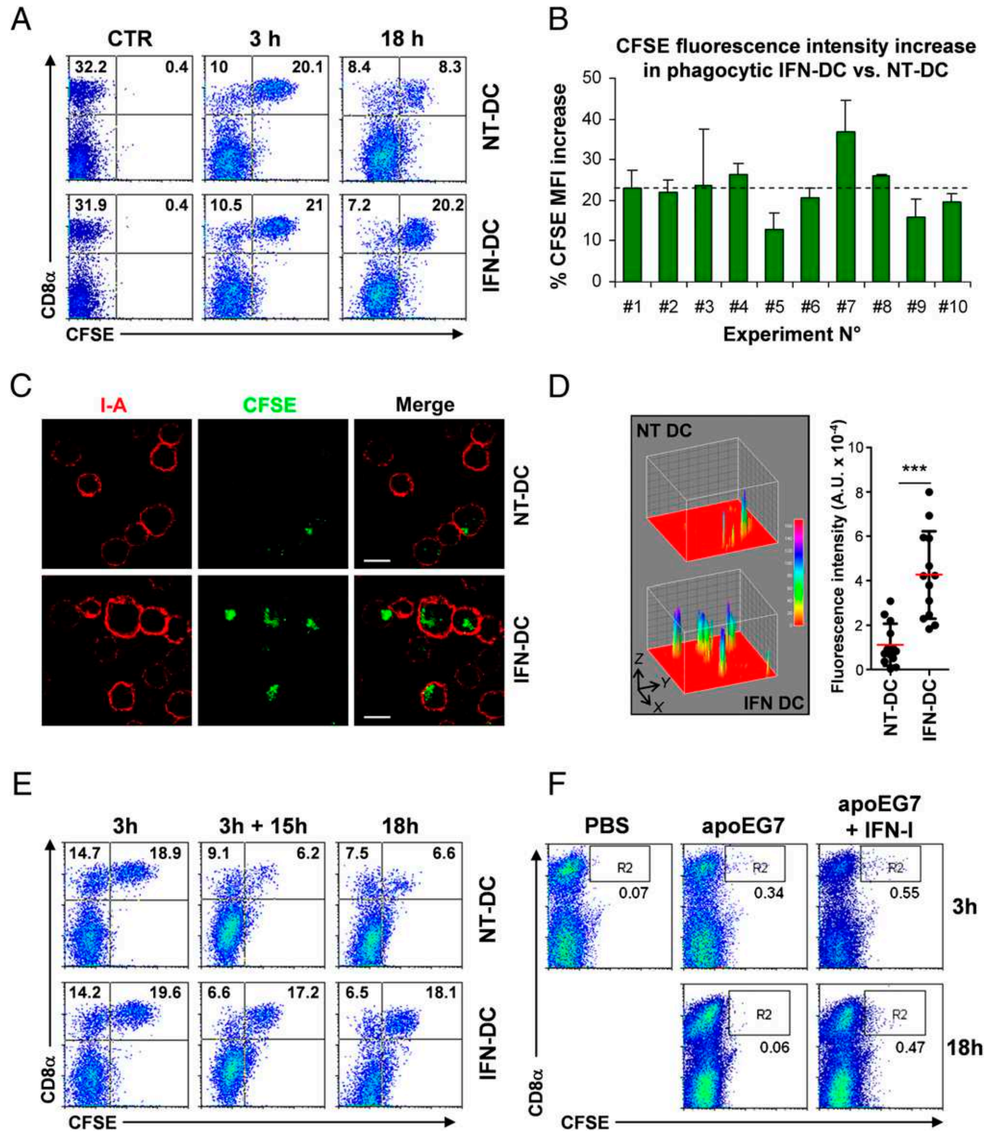

FIGURE 2. IFN-I prolong Ag persistence after phagocytosis of apoEG7 cells by CD8α$^+$ DC and regulate intracellular pH. *A*, Magnetically sorted splenic CD11c$^+$ DC were cocultured in the presence or absence of IFN-I (5×10^3 U/ml) with CFSE-labeled apoEG7 cells at 4˚C (CTR), or at 37˚C for 3 h (3h) or 18 h (18h), then separated from apoptotic cells by Nycodenz centrifugation, surface stained for CD8 and CD11c expression, and analyzed by FACS. Plots represent the percentage of CD11c$^+$-gated DC engulfing apoEG7 cells (CFSE$^+$). One representative experiment of six is shown. *B*, Analysis of CFSE MFI in phagocytic DC (CD11c$^+$CD8α$^+$CFSE$^+$ gated), IFN-treated versus untreated. Plot represents the percentage increase of MFI in IFN-DC with respect to NT-DC of each individual experiment ± SD. Dotted line depicts the mean value of 10 experiments. *C*, CD11c$^+$ DC cocultured with CFSE$^+$ apoEG7 cells (green) ± IFN-I for 18 h were labeled with I-A Ab (red) and analyzed by CLSM. Several cells for each labeling condition were analyzed, and representative images are shown. Scale bars, 10 μm. One representative experiment of two is shown. *D*, Confocal images were analyzed using ImageJ software to map the integrated fluorescence intensity for single CFSE$^+$ cells, as a parameter depicting the amount of Ag per cell, and the resulting three-dimensional histograms are shown (*left panel*). The mean integrated fluorescence intensity values (in arbitrary units) ± SD of all cells analyzed (n = 15) from several images per condition (NT-DC and IFN-DC) are also indicated (*right panel*). *E*, CD11c$^+$ DC were cocultured with CFSE$^+$ apoEG7 cells ± IFN-I for 3 h (3h), or separated at 3 h from apoptotic cells and then replated alone for additional 15 h (3h + 15h), or left in coculture 18 h continuously (18h) and then analyzed by FACS for the presence of intracellular apoEG7 cells. Plots show a population gated on CD11c positivity and are representative of one experiment of three. *F*, C57BL/6 mice (n = 5 mice per group) were injected i.v. with CFSE-labeled apoEG7 cells alone or in combination with 10^6 U IFN-I. Three and 18 h later, splenic DC were magnetically sorted and uptake was evaluated by FACS analysis on gated CD11c$^+$ cells. One experiment of three is shown.

in Ag retention. As expected, DPI also decreased the proportion of CFSE$^+$CD8α$^+$ DC in cultures not containing IFN-I (Fig. 3*B*). These data strongly suggest that the effects of IFN-I on Ag retention by CD8α$^+$ DC can involve a regulatory mechanism of pH within phagosomal vesicles.

IFN-I sustain the survival of Ag-bearing CD8α$^+$ DC

In addition to the effects on Ag retention, we found that IFN-I treatment resulted in increased numbers of total CD8α$^+$ DC recovered after 18-h coculture with apoEG7 cells, as revealed by percentages in IFN-I–containing cultures with respect to untreated cultures (26.7 versus 15.3%; Fig. 4*A*), and by absolute numbers of CD8α$^+$ DC retrieved in the cultures (Fig. 4*B*). These observations suggest that IFN-I could also promote the survival of Ag-bearing CD8α$^+$ DC, namely DC that have engulfed apoEG7 cells. To test this, we analyzed the mortality of sorted CD8α$^+$ and CD8α$^-$ DC after culture with CFSE$^+$ apoEG7 cells in the presence or absence of IFN-I. As revealed by PI staining, addition of IFN-I significantly decreased the percentage of dying CD8α$^+$ DC (32.5%), with respect to untreated cells (50.3%; Fig. 4*C*). Conversely, IFN-I

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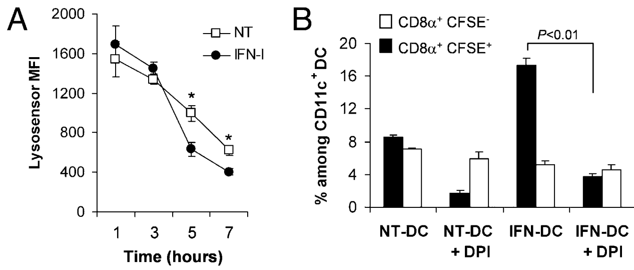

FIGURE 3. IFN-I affects intracellular pH of phagocytic CD8α+ DC. *A*, DC cocultured with apoEG7 cells ± IFN-I were harvested and labeled with allophycocyanin anti-CD11c and Lysosensor green (5 μM) at the indicated times of coculture. Each point represents Lysosensor MFI in CD11c+-gated cells of triplicate samples (mean ± SD). $*p < 0.05$. One experiment of three is shown. *B*, DC were cocultured with CFSE-labeled apoEG7 cells ± IFN-I for 3 h, after which DPI (5 μM) was added where indicated for the remaining 15-h culture. Data indicate the mean percentage of CFSE+ and CFSE− cells among gated CD11c+CD8α+ DC at 18 h in triplicate cultures + SD. One representative experiment of two is shown.

caused increased mortality levels of CD8α− DC (61.5%), with respect to untreated controls (37.5%; Fig. 4*C*), in keeping with the reduced numbers of CD8α− DC retrieved in the cultures containing IFN-I (Fig. 4*B*). Of note, among CD8α+ DC, IFN-I selectively promoted the survival of phagocytic cells, as revealed by reduced PI staining in CD8α+CFSE+ cells (27.8%) with respect to untreated controls (60.2%), but not that of nonphagocytic cells, as revealed by similar percentages of PI+ cells in IFN-treated versus untreated CD8α+CFSE− (41.1 versus 47.1%; Fig. 4*D*). Because we previously reported that IFN-I promote apoptosis of bystander DC, such as DC not bearing Ag, by downmodulating antiapoptotic molecules of the bcl-2 family (15), we analyzed whether IFN-I could modulate the expression of these genes in Ag-bearing CD8α+ DC. As illustrated in Fig. 4*E*, IFN-I treatment markedly increased the expression of bcl-2 and bcl-x_L in CD8α+ DC after 3- and 18-h culture. In contrast, IFN-I treatment decreased both bcl-2 and bcl-x_L in CD8α− DC, to be considered as bystander DC in this setting (Fig. 4*E*). These results confirm our previous findings on the proapoptotic effects of IFN-I on bystander DC (15) and suggest that these cytokines may instead act as a survival factor for Ag-bearing DC.

IFN-I promote cross-presentation of cell-associated OVA by CD8α+ DC and stimulate CD8 T cell priming

Previous studies have shown that the expression of SIINFEKL peptide of OVA in association with MHC-I (MHC-OVAp) on DC membrane can be highly increased by LPS treatment, suggesting that signals capable of activating DC could also enhance cross-presentation (22). Thus, to assess whether IFN-I affected cross-presentation of apoEG7-derived antigenic material, we analyzed MHC-OVAp surface expression on CD8α+ DC by FACS. Remarkably, IFN-I strongly enhanced the levels of MHC-OVAp in CFSE+CD8α+ DC, with respect to untreated cells (49.7 versus 8.7% expressing cells; Fig. 5*A*). Of note, IFN-I–induced upregulation of MHC-OVAp expression on CD8α+ DC was selectively abolished by blocking with Ab to mouse IFN-I, demonstrating that this effect was specifically mediated through IFN-I receptor (Fig.

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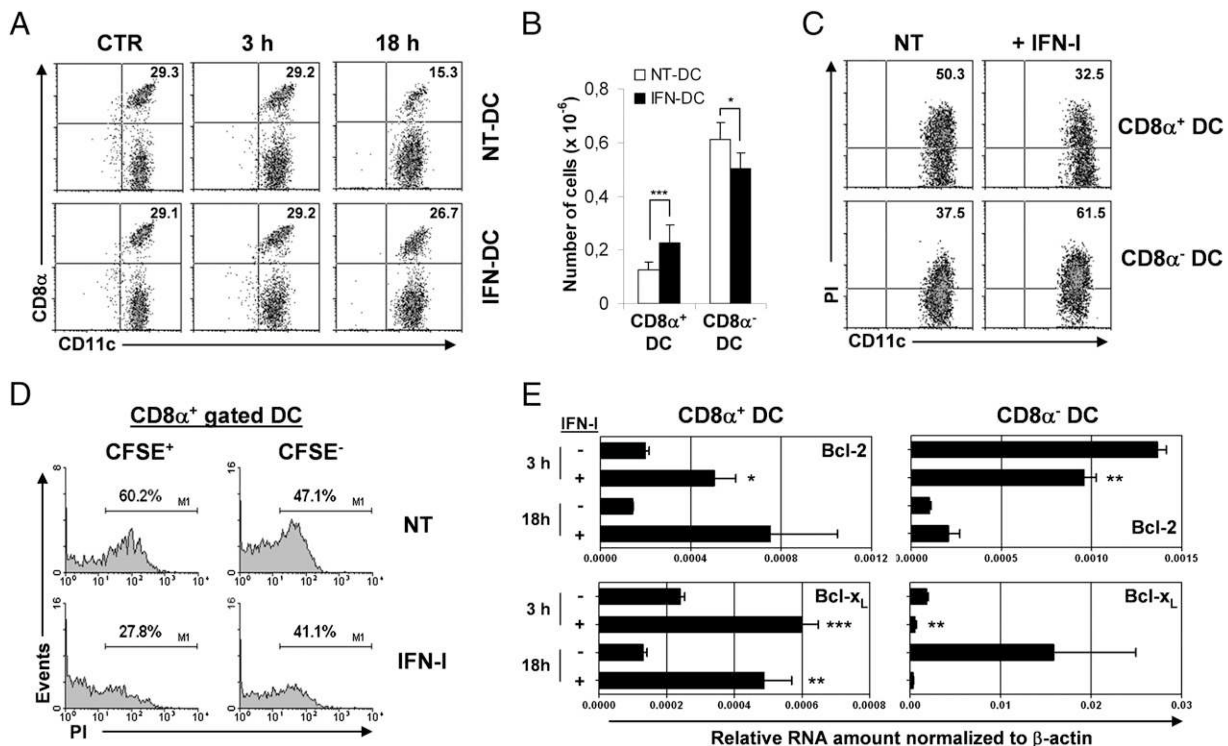

FIGURE 4. IFN-I promote the survival of phagocytic CD8α+ DC by selective upregulation of antiapoptotic genes. *A*, Splenic DC were cocultured with CFSE-labeled apoEG7 cells ± IFN-I at 4°C (CTR), or at 37°C for 3 or 18 h, then analyzed by FACS. Plots represent the percentage of CD8α+ DC in a population of living cells gated based on forward–side scatter properties. One representative experiment of six is shown. *B*, Enumeration of CD8α+ and CD8α− DC after coculture for 18 h with apoEG7 cells ± IFN-I. Data represent the mean values of 12 separate experiments ± SD. $*p < 0.05$, $***p < 0.001$. *C*, Sorted CD8α+ and CD8α− DC were cocultured with CFSE+ apoEG7 cells in the presence or absence of IFN-I. Eighteen hours later, cells were analyzed by FACS for PI staining. *D*, PI staining in CFSE+- and CFSE−-gated CD8α+ DC. *E*, DC were sorted into CD8α+ and CD8α− DC after 3- or 18-h coculture with apoEG7 cells ± IFN-I. RNA was extracted and subjected to quantitative RT-PCR analysis for bcl-2 and bcl-x_L expression. Plots represent the relative amount of mRNA normalized to β-actin run in triplicate ± SD. One representative experiment of three is shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

5*A*). Moreover, expression of MHC-OVAp in IFN-treated phagocytic CD8α$^+$ DC was also inhibited by DPI, supporting the involvement of intraphagosomal pH regulation by IFN-I in stimulating cross-presentation (Fig. 5*A*). To become competent for cross-priming, DC require a license signal inducing full maturation. In this regard, IFN-I have been widely described to activate DC for induction of Ag-specific T and B cell immunity (23, 24). Thus, we analyzed whether IFN-I exposure resulted also in activation of CD8α$^+$ DC that had taken up apoEG7 cells and found noticeable activation of these cells, as revealed by increased expression of CD40 and CD86 molecules with respect to untreated cells (Fig. 5*B*). Of note, IFN-I induced far more marked phenotypic activation in phagocytic CD8α$^+$ DC, with respect to nonphagocytic CD8α$^+$ DC in vitro (Fig. 5*B*) and in vivo, when injected in combination with apoEG7 cells (Fig. 5*C*).

To investigate the ability of IFN-treated CD8α$^+$ DC to cross-prime CD8 T cells against cell-associated OVA, we cultured DC loaded with apoEG7 in the presence or absence of IFN-I with CFSE-labeled OT-I lymphocytes and analyzed OVA-specific responses. Consistent with the increased levels of cross-presented OVA, IFN-treated DC stimulated higher proliferation of OT-I cells, as compared with untreated controls, again indicating increased OVA cross-presentation (Fig. 6*A*). Of interest, OT-I CD8 T cells responding to IFN-DC, unlike those responding to NT-DC, exhibited an activated phenotype, as revealed by expression of CD25 and CD69 (Fig. 6*A*). Cross-priming of OT-I cells in response to IFN-treated DC was also confirmed by IFN-γ ELISPOT assay (Fig. 6*B*). Remarkably, even IFN-DC to whom apoEG7 cells were withdrawn after 3-h coculture (IFN-DC 3 h + 15 h; Fig. 6*B*) were potent stimulators of IFN-γ–specific effector response, in accordance with their mature phenotype (Supplemental Fig. 1), priming OT-I cells as efficiently as IFN-treated DC from the continuous coculture (IFN-DC 18 h; Fig. 6*B*). In contrast, apoEG7-DC alone induced poor OT-I effector response (NT-DC 18 h; Fig. 6*B*). Next, we examined the capacity of IFN-treated DC to induce CD8 T cell cross-priming in vivo when injected into naive C57BL/6 mice adoptively transferred with CFSE-labeled OT-I cells. In an attempt to use as stimulators apoEG7-loaded DC generated by 18-h coculture with or without IFN, we failed to observe measurable proliferative responses in vivo (data not shown). Thus, we immunized mice by injecting apoEG7-DC (i.e., DC loaded with apoEG7 cells by 3-h coculture) alone or combined with IFN-I, so as to prolong cytokine exposure in vivo. Three days later, we measured OVA-specific T cell priming in draining or, as a control, distal LN by FACS. Remarkably, only mice immunized with apoEG7-DC plus IFN-I displayed sustained proliferative response in draining LN (Fig. 7*A*), resulting also in activation of OT-I cells, as evidenced by CD25 upregulation in proliferating cells (11.5%; Fig. 7*B*). In contrast, mice injected with apoEG7-DC alone failed to induce OT-I proliferation in vivo, confirming the tolerogenic potential of these cells (Fig. 7*A*). As expected, no significant OT-I cell response could be detected in distal LN from mice immunized with apoEG7-DC plus IFN-I (Fig. 7*A*, 7*B*). Lastly, we examined the ability of IFN-I to license DC for cross-priming in vivo by injecting apoEG7 cells, alone or combined with IFN-I, in wild-type (WT) C57BL/6 or IRF-8$^{-/-}$ mice, whose DC are unable to capture apoEG7 cells. Strikingly, IFN-stimulated OT-I cross-priming was detected in WT, but not in IRF-8$^{-/-}$ recipients, as revealed by substantial proliferation of adoptively transferred OT-I lymphocytes in mice injected with apoEG7 plus IFN-I (70%; Fig. 7*C*), indicating that the cytokines were mediating this effect through CD8α$^+$ DC stimulation.

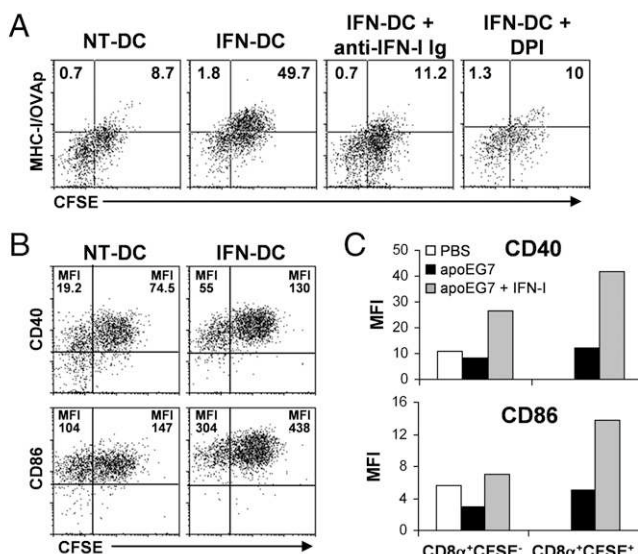

FIGURE 5. IFN-I enhance cross-presentation of cell-associated OVA by CD8α$^+$ DC. *A* and *B*, Phenotype of CD11c$^+$CD8α$^+$ splenic DC after 18-h coculture with CFSE-labeled apoEG7 cells in the absence (NT-DC) or presence of IFN-I (IFN-DC), of IFN-I plus sheep anti–IFN-I Ig (IFN-DC + anti–IFN-I Ig), or of IFN-I plus DPI (IFN-DC + DPI). *A*, Analysis of SIINFEKL peptide–MHC-I complex (MHC-I–OVAp) expression. Plots depict the percentage of cells in *upper left* (CFSE$^-$) and *upper right* (CFSE$^+$) *quadrants*. *B*, Analysis of CD40 and CD86 expression in gated CD8α$^+$CD11c$^+$ cells. MFI values in each plot refer to *y*-axis in *upper right* and *upper left quadrants*. One experiment of five is shown. *C*, C57BL/6 mice (n = 4 mice per group) were i.v. injected with CFSE$^+$ apoEG7 alone or in combination with either IFN-I or PBS. Three hours later, mice were sacrificed and splenic DC were FACS analyzed. Histograms represent the expression levels of CD40 and CD86 in CD11c$^+$-gated CD8α$^+$CFSE$^+$ and CD8α$^+$CFSE$^-$ DC. One experiment of three is shown.

Discussion

Cells dying purposefully by apoptosis are thought to be phagocytosed by mechanisms that fail to incite inflammatory or immune reactions. Hence, clearance of apoptotic cells by phagocytes results in anti-inflammatory and immunosuppressive effects, thus hampering the onset of T cell effector responses. This occurs because engulfment of apoptotic material results in lack of induction of proinflammatory cytokines or even in the release of immunoregulatory factors that maintain DC in an immature state (25). The findings reported in this study demonstrate that IFN-I can act as a powerful switch signal for DC promoting cross-priming in vivo against a largely tolerogenic type of Ag, such as Ag derived from tumor apoptotic cells. In doing so, IFN-I control CD8α$^+$ DC activity at three distinct levels. First, IFN-I treatment prolongs the intracellular persistence of antigenic particles engulfed by phagocytic CD8α$^+$ DC, as revealed by increased levels of CFSE fluorescence intensity in phagocytic CD8α$^+$ DC after 18-h culture. As a result, IFN-treated DC exhibited enhanced cross-presentation of apoptotic cell-derived OVA, as evidenced by surface expression of MHC-I–OVAp complexes and by induced OT-I cell proliferation. Ag persistence is a crucial event regulating the magnitude of cross-presentation and is promoted by a reduced lysosomal proteolysis that delays the degradation of phagocytosed Ag, in a process requiring a limited phagosomal acidification. As a mechanism regulating intraphagosomal pH, the NOX2 enzyme was shown to induce active alkalinization of the phagolysosomal compartments selectively in DC (7). In this study, Ag persistence induced by IFN-I strongly correlated with pH alkalinization and was restrained by addition of the NOX2 inhibitor DPI, resulting in

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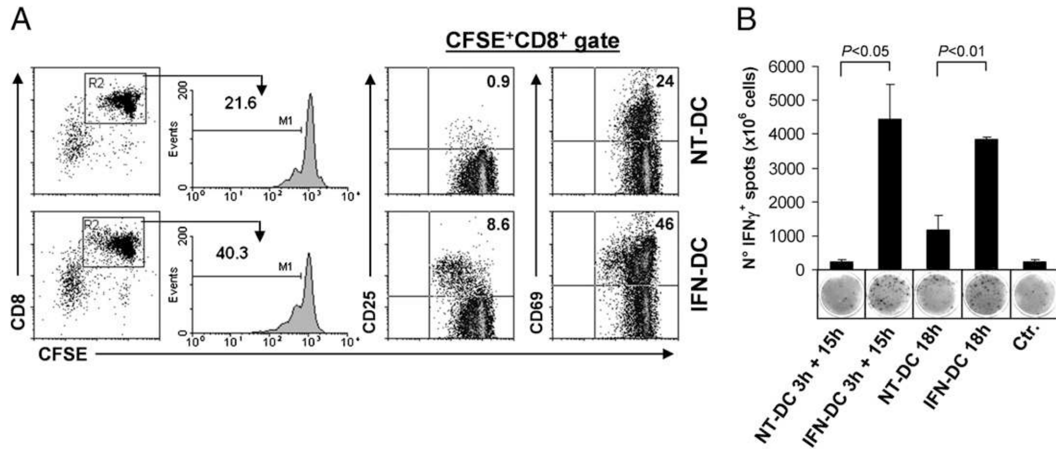

FIGURE 6. IFN-I promote CD8 T cell cross-priming against cell-associated OVA in vitro. *A*, NT-DC and IFN-DC were cocultured with CFSE-labeled OT-I T cells for 3 d. Histogram plots represent CFSE dilution profiles in gated CD8+CFSE+ T cells. Density plots represent the percentage of gated CD8+ CFSE+ T lymphocytes expressing CD25 and CD69. Data are representative of three separate experiments. *B*, IFN-γ release by OT-I T cells responding to NT-DC, IFN-DC, NT-DC 3 h + 15 h, or IFN-DC 3 h + 15 h (as in Fig. 2*C*), measured by IFN-γ ELISPOT assay, as described in *Materials and Methods*. Histograms represent numbers of IFN-γ–forming spots in each triplicate sample (mean + SD). One representative well is depicted.

reduced cross-presentation of EG7-derived OVA by CD8α+ DC. Previous studies reported that signaling through TLR2, TLR3, TLR4, and TLR9 enhances Ag uptake, resulting in more efficient cross-presentation (26). In the current study, we show that IFN-I enhance CD8α+ DC cross-presentation of tumor apoptotic cell-derived Ag mainly affecting Ag processing. Consistent with this view, studies on human DC indicate that IFN-I can affect the

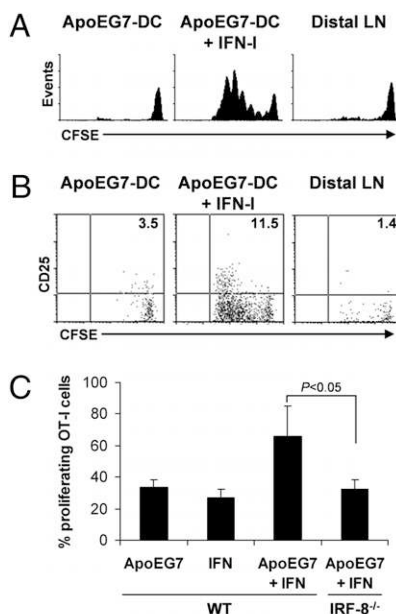

FIGURE 7. IFN-I promote CD8 T cell cross-priming against cell-associated OVA in vivo. *A* and *B*, C57BL/6 mice (*n* = 4 mice per group) adoptively transferred with CFSE-labeled OT-I cells were immunized in the footpad with apoEG7 loaded DC ± IFN-I. Three days later, OVA-specific CD8 T cell response was measured in popliteal LN or distal LN. *A*, Proliferation of transferred OT-I cells, as measured by CFSE dilutions. *B*, Percentage of CD25-expressing CD8+CFSE+ T cells. The results of one representative experiment of three are shown. *C*, IRF-8$^{-/-}$ or WT C57BL/6 mice (*n* = 4 mice per group) adoptively transferred with CFSE-labeled OT-I cells were injected i.v. with apoEG7 cells ± IFN-I, or IFN-I alone. Three days later, OVA-specific proliferative response was measured in the spleen. Plots represent the percentages of proliferating OT-I cells in each individual mouse (mean + SD). The data are representative of one of three experiments performed under identical experimental conditions.

expression of a number of genes associated with processing as well as the expression of inducible proteasome subunits (11, 27, 28). It is worth mentioning that in our setting, withdrawal of apoEG7 cells from the coculture at 3 h did not prevent IFN-induced Ag retention and OVA cross-presentation in CD8α+ DC, provided that IFN-I were maintained in the culture for the remaining 15 h. In fact, removal of both apoEG7 cells and IFN-I after the 3-h culture resulted in only partial Ag retention and no DC activation and cross-presented OVA (Supplemental Fig. 2). This observation suggests that IFN-I exposure may be required all through the Ag-processing phase to lead to MHC-I cross-presentation and DC activation.

As a second effect, IFN-I promoted the survival of phagocytic, but not of nonphagocytic, CD8α+ DC, through the selective modulation of the apoptosis-related genes Bcl-2 and Bcl-x_L. This finding, as opposed to our previous finding showing IFN-I exerting proapoptotic effects on bystander (i.e., in the absence of Ag) DC, suggests an elegant regulatory mechanism by which IFN-I selectively sustain the life span of Ag-bearing DC for induction of effective immune responses, while favoring a rapid clearance of steady-state DC (15). The duration of DC life span critically regulates the efficiency of cross-priming and the outcome of adaptive immunity, although little is known about the role of Ag persistence in this process (29). A recent study in mice infected with bacillus Calmette-Guérin has shown that Ag persistence in infected DC is strictly correlated with DC survival (30). Thus, these data suggest that duration of Ag persistence and survival of DC may be two linked processes regulating the extent of Ag presentation and cross-presentation. In this respect, the effects of IFN-I in promoting both Ag persistence and survival of CD8α+ DC may be regarded as two tightly correlated events, because in our studies increased Ag retention by the CD8α+ DC strongly correlated with a longer life span of these cells, although further investigations are required to determine which process is causative of the other.

A third process accounting for IFN-I effects is the activation of DC, revealed by upregulation of both costimulatory markers and proinflammatory cytokines (Supplemental Fig. 3), that provide a license signal for DC to cross-priming, consistent with previous reports showing IFN-I to be a powerful stimulus for DC activation (9, 24). Multiple events have been described licensing DC for cross-priming that include CD40L engagement by CD4 Th cells,

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stimulation by NK cells, TLR triggering, and exposure to soluble factors released upon injury or infection (5, 31). Among these soluble mediators, IFN-I have been described to be particularly efficient in inducing cross-priming in a CD4 T cell-independent manner, implying a faster immune reaction (9, 10). Besides the appreciated effects in promoting cross-priming against soluble or viral Ag, some recent evidence suggests that IFN-I may also affect cross-presentation of cell-associated Ag (11). Of note, a recent study on the newly described mouse merocytic DC subset has shown that these cells are endowed with potent ability to prime both CD4 and CD8 T cells against tumor cell-associated Ag partly through their ability to produce IFN-I upon engulfment of apoptotic tumor cells (32). Moreover, cellular association of dsRNA with irradiated EG7 cells was shown to elicit CD8 T cell responses in vivo that were dependent on dsRNA-induced IFN-I secretion by DC (33). Finally, we have recently reported that IFN-I can greatly enhance cross-presentation and CD8 T cell cross-priming by stimulating CD8α^+ DC that have engulfed tumor cells undergoing an immunogenic type of apoptosis by chemotherapy treatment (13). The present study extends this view to demonstrate that IFN-I can act as a switch signal for CD8α^+ DC cross-presenting tumor apoptotic cell-derived Ag converting the response into cross-priming.

The property of innate stimuli, such as those triggering TLR3, TLR4, and TLR9, to stimulate T cell responses has been shown to be largely dependent on the induction of endogenous IFN-I (34–36). Of interest, TLR agonists have been shown to stimulate cross-priming either at the level of DC licensing or by enhancing Ag cross-presentation (37, 38). Our results suggest that IFN-I induce cross-priming stimulating both processes in CD8α^+ DC and, additionally, by sustaining the life span of Ag-bearing DC. Because mouse CD8α^+ DC are specialized for MHC class I presentation and CD8 T cell activation, these cells are regarded as the ideal DC subtype for targeted vaccination to generate effector CTL responses. Given the recent discovery of human equivalents of mouse CD8α^+ DC (39–42), our studies provide new knowledge on IFN-I properties to be exploited for the design of innovative clinical protocols in which the generation of effective cytotoxic immunity is crucially required, such as in anticancer treatments. Importantly, a role for IFN-I in induction of autoimmunity has recently emerged. In fact, therapeutic treatment with IFN-I, especially IFN-α, in cancer and other pathologies has been associated with the onset of collateral autoimmune disorders, leading to the hypothesis that these effects may be due to a hyperstimulation of immune cells, such as DC, by these cytokines (43). The results reported in this study support this concept and provide a potential mechanism by which IFN-I may induce autoimmune reactions, namely through the enhancement of DC activation and the presentation of self Ag derived from cells undergoing constitutive apoptosis.

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Disclosures

The authors have no financial conflicts of interest.

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REVIEW

Immunomodulatory effects of cyclophosphamide and implementations for vaccine design

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Abstract Drug repositioning refers to the utilization of a known compound in a novel indication underscoring a new mode of action that predicts innovative therapeutic options. Since 1959, alkylating agents, such as the lead compound cyclophosphamide (CTX), have always been conceived, at high dosages, as potent cytotoxic and lymphoablative drugs, indispensable for dose intensity and immunosuppressive regimen in the oncological and internal medicine armamentarium. However, more recent work highlighted the immunostimulatory and/or antiangiogenic effects of low dosing CTX (also called "metronomic CTX") opening up novel indications in the field of cancer immunotherapy. CTX markedly influences dendritic cell homeostasis and promotes IFN type I secretion, contributing to the induction of antitumor cytotoxic T lymphocytes and/or the proliferation of adoptively transferred T cells, to the polarization of $CD4^+$ T cells into TH1 and/or TH17 lymphocytes eventually affecting the Treg/Teffector ratio in favor of tumor regression. Moreover, CTX has intrinsic "pro-immunogenic" activities on tumor cells, inducing the hallmarks of immunogenic cell death on a variety of tumor types. Fifty years after its Food and Drug Administration approval, CTX remains a safe and affordable compound endowed with multifaceted properties and plethora of clinical indications. Here we review its immunomodulatory effects and advocate why low dosing CTX could be successfully combined to new-generation cancer vaccines.

Keywords Cyclophosphamide · Chemotherapy · Immunotherapy · Cancer vaccine · Immunomodulation · Cancer

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Introduction

Attractive developments are currently emerging in clinical oncology, based on the assumption that to win the fight against cancer, it is necessary not only to kill malignant cells but also to hijack the host immune system so that it controls residual disease. Working at the frontier between cancer immunology, genetics, and cell biology may integrate parameters pertaining to the host–tumor interactions unraveling novel avenues to harness cancer.

There is accumulating evidence that the success of conventional therapies against cancer may stem, at least in part, from the activation of the host immune system. This immune contribution can be elicited in three ways by the current therapies. First, a selected panel of drugs can induce immunogenic tumor cell death in that specific danger signals become emitted by dying or stressed cells upon exposure to the cytotoxic compounds [1]. Secondly, beyond their effect on the tumor itself, some drugs may indirectly stimulate distinct immune components, by inducing a transient lymphodepletion, by subverting immunosuppressive mechanisms, or by directly stimulating immune effectors. Thirdly, many experimental therapies that have been tested in mice and humans suggest that vaccination against cancer-specific antigens can sensitize tumor cells to chemotherapy-induced apoptosis, at least in part through induction of the mannose-6-phosphate receptors which promotes a perforin-independent increase in permeability to granzyme B released by cytotoxic T lymphocytes (CTL) [2]. The emphasis on the immunomodulatory potential of chemotherapy challenges the traditional perception that chemotherapy and immunotherapy act through unrelated, supposedly antagonistic mechanisms.

Cyclophosphamide (*N*,*N*-bis (2-chloroethyl)-1, 3, 2-oxazaphosphinan-2 amine 2-oxide, the generic name for Cytoxan° (CTX), Endoxan°) is a nitrogen mustard alkylating agent from the oxazophorine group. It was developed by Norbert Brock and ASTA company [3]. In 1958, CTX was first assayed in clinical trials for the treatment of cancer [4, 5], and in 1959, it became the eighth cytotoxic anticancer agent approved by the Food and Drug Administration (FDA). Fifty years after its synthesis, CTX remains one of the most successful and widely used drugs for the treatment of a variety of diseases, including hematological and solid malignancies and autoimmune disorders, and as a conditioning regimen for blood and marrow transplantation and stem-cell mobilization. However, the biological activities of CTX are dose dependent. In 1988, Robert North and, later on in 2000, Judah Folkman pioneered the concept of "metronomic dosing" of alkylating agents (10–40 mg/kg in mice, 50 mg/day per os or about 250 mg/m^2 iv in humans) where the immunostimulatory and antiangiogenic attributes of CTX were best highlighted, paving the way to the combination therapies admixing novel immunomodulatory or antiangiogenic compounds together with low-dose CTX with objective clinical success [6–10]. It is unclear how convergent and/or overlapping are the two biological effects obtained with metronomic dosing of CTX. Indeed, CTX-induced IFN type I and type II as well as TH1 cells may well contribute to the antiangiogenic properties of CTX. Nevertheless, in this review, we will summarize the main mechanisms by which CTX mediates its immunostimulatory effects unraveled in preclinical models (induction of a TH2/TH1−TH17 shift in cytokine production [11, 12], reduction of tumor-induced suppressor T cell frequencies [13], enhancement of long-term survival and proliferation of lymphocytes [14, 15], induction of a variety of soluble mediators [16], resetting of dendritic cell (DC) homeostasis) [17–22] (Schiavoni et al. 2010) and discuss the encouraging results obtained in recent clinical trials utilizing CTX combined with tumor vaccines.

Cyclophosphamide and B cells

So far, cancer vaccines primarily aimed at stimulating the cellular arm of immunity, i.e., T cell responses directed against tumor-associated antigens (TAA) [23]. Indeed, B cells have always been considered as negative regulators of T cell-dependent tumor immunosurveillance [24–26]. In the K14-HPV16 mouse model of squamous carcinogenesis, humoral immunity and B cells foster cancer development by attracting FcγR expressing myeloid cells that interact with antibodies residing in the stroma of premalignant lesions. Therapies targeting these pro-tumoral B cells (such as Rituximab) synergize with cytotoxic agents [27]. New B cell subsets, namely Breg, may mediate peripheral tolerance by producing IL-10 [28, 29]. However, passive immunotherapy with TAA-specific monoclonal antibodies (mAb) showed some clinical benefit [30], but the limited half-life and the compromised pharmacological availability of passively transferred antibodies limited such approaches. Interestingly, distinct antibody responses raised against certain antigenic specificities might be of therapeutic interest. Indeed, the group of Dranoff showed that granulocyte macrophage-colony stimulating factor (GM-CSF) engineered tumor vaccines elicit anti-MICA/B antibodies that may interfere with the immunosuppressive effects of soluble MICA and/or promote cross-presentation of tumor antigens through FcγR-bearing antigen-presenting cells (APC) [31, 32]. Similar findings were reported with anti-NY-ESO-1 Ab [33, 34]. Importantly, antibodies directed against an oncogenic receptor (such as HER-2) may be of great therapeutic value, as shown by Forni's group using different types of vaccine formulations [35].

Before the introduction of Rituximab, CTX was the lead compound to deplete peripheral B cells. In the late 1980s, Zhu et al. [36] carried out a pilot study on a cohort of 12 patients with non-neoplastic immune-mediated diseases assessing the effects of long-term exposure to low-dose CTX (2 mg/kg per day). They reported B cell defects at multiple levels, i.e., B cell activation, proliferation, and differentiation. Chronic exposure to low-dose CTX reduced serum antibody levels. These effects were transient with full recovery after CTX discontinuation. In the subsequent decade, the group of Proietti and colleagues made signif-

icant breakthroughs in the understanding of the CTX-mediated antitumor effects when combining CTX to adoptive transfer of spleen-derived immune cells [14, 15]. After CTX (100 mg/kg)-induced lymphodepletion, a "rebound" phase occurs, during which a cytokine storm drives the homeostatic proliferation, activation, and trafficking of different lymphocyte pools, including B cells [16]. This phenomenon was not as prominent in severe combined immunodeficiency (SCID) mice (where "space" was also available for expansion) and was associated with a "window" (from 5 h to day 3 post-CTX) of lymphopenia generating high levels of endogenous IL-7. Interestingly, CTX induced the homing of transferred B cells to secondary lymphoid organs more specifically when the lymphocytes were derived from immunized rather than naïve donor mice. The higher number of transferred $CD19^+$ B cells recovered post-CTX correlated with elevated and sustained levels of tumor-specific serum antibodies not seen in phosphate-buffered saline-treated mice. In addition, CTX-induced high serum IgG titers appeared to correlate with the cure of animals (Sestili, unpublished observations). More recently, Montero and coworkers [37] reported that humoral immune responses to an epidermal growth factor-based cancer vaccine in Montanide could be significantly boosted by high-dose CTX.

Cyclophosphamide and DC cross-presentation

The induction of an effective antitumor response requires the active participation of APC such as specific DC subsets [38] responsible for optimal cross-presentation of exogenous antigens into the MHC class I pathway and efficient T lymphocyte priming [39, 40]. Recently, the human equivalent of the mouse $CD8\alpha^+$ DC [41–43], mostly eligible for cross-priming and cross-tolerance, is the $BDCA3^+/CD141^+$ DC on which rely future cell vaccine developments [44–46].

Accumulating evidence points to the capacity of CTX to mobilize bone marrow (BM) DC [19, 22, 47]. Salem and coworkers [19] showed that CTX-induced lymphodepletion is responsible for a marked expansion of immature DC in peripheral blood, peaking on day 12 post-CTX (during the so termed "rebound or restoration" phase). The expanded DC significantly contributed to the beneficial effects of CTX to adoptive T cell therapy, since their depletion reduced the antigen specific accumulation of adoptively transferred $CD8^+$ T cells. By sparing BM DC precursors, CTX administration facilitated the recovery of an immature DC pool in the periphery contributing to enhanced T cell priming in vivo [22]. However, at higher dose (200 mg/kg), CTX depleted BM DC precursors in tumor-bearing mice, thus supporting the concept of a dose-dependent sensitivity of these progenitors to chemotherapy [47]. Moreover, CTX could influence the quality of the peripheral DC pool by modulating the balance in-between DC subsets in secondary lymphoid organs [21, 22]. Two groups reported a selective ablation of $CD8\alpha^+$ resident DC in both spleen and lymph nodes, known to participate in peripheral tolerance. Hence, the pool of $CD11c^+$ DC became more potent IL-12 producing antigen-presenting cells post-CTX than in untreated mice [21, 22].

Interestingly, CTX can operate direct changes on dying tumor cells, contributing to their immunogenicity before cell death occurs. We have recently shown that CTX administration in tumor-bearing mice induces pre-apoptotic surface translocation of calreticulin (ecto-CRT) on tumor cells [22], which serves as an "eat-me" signal for phagocytes [48] and the release of high-mobility group box1 protein in the extracellular milieu [22], which constitutes a "danger signal" triggering activation of the DC processing machinery [49]. These events are prerequisites for adequate engulfment of tumor apoptotic material and optimal $CD8^+$ T cell cross-priming by DC [48, 49].

Cyclophosphamide and TH1

Immune responses are regulated by several subtypes of $CD4^+$ helper T cells, including TH1 and TH2, which produce two types of cytokines with markedly different properties. While type-1 cytokines (IL-2, IFNγ, etc.) are involved in cell-mediated immune reactivities which are essential for an effective antitumor activity, type-2 cytokines (IL-4, IL-5, IL-6, and IL-10) are commonly found in association with strong humoral responses [50]. TH1 and TH2 responses are known to be cross-regulated in a reciprocal manner through IFNγ which inhibits TH2 cells [51] and IL-10 which inhibits TH1 cells [52]. Based on this scheme, different therapeutic modalities have been proposed to promote a cytokine shift generating favorable antitumor cellular responses.

Matar et al. [11] described a TH2/TH1 shift in cytokine production of tumor-bearing rats treated with low-dose CTX. This study clearly showed that spleen cells from tumor-bearing rodents secrete low amounts of IFNγ and IL-2 at baseline but significantly enhanced levels post-CTX [11]. Conversely, the levels of type-2 cytokines (i.e., IL-10) and suppressive agents such as TGF-β and NO produced by spleen cells significantly increased in tumor-bearing rats pre-CTX, decreased after CTX treatment [53]. Indeed, Proietti's group has extensively studied the modulations of the homeostatic equilibrium in different hematopoietic and immune compartments (BM, LN, tumor) reporting the preferential expansion and persistence of anti-tumor T cells

[14–16]. In various tumor models, a single ip injection of CTX markedly enhanced the antitumor efficacy of an adoptive transfer of splenocytes (from tumor-bearing mice). Indeed, CTX primed the host through the induction of bystander effects (i.e., production of growth factors normally occurring as a homeostatic response post-chemotherapy) which promoted proliferation, survival, and activation of $CD4^+$ TH1 lymphocytes proved to be the key players in the adoptive transfer [14, 16]. Intriguingly, the adoptive transfer of immune splenocytes into immunocompetent mice treated with CTX was far more efficient than the adoptive transfer operated in an "empty space" such as SCID counterparts (not treated with CTX). It is conceivable that the "cytokine storm" (where IL-7 played an important part) induced by day 2 post-CTX contributed to the TH1 polarization and expansion [14]. In fact, such a chemoimmunotherapy regimen (CTX plus immune cells) significantly increased plasma levels of IFNγ compared with controls, while decreasing that of IL-10. Notably, among CTX-induced cytokines, IFN type I are major soluble factors mediating many of the effects ascribed to the drug, such as the polarization toward a TH1 type of immune response [54] and the expansion of $CD4^+$ and $CD8^+$ T cells exhibiting a memory ($CD44^{hi}$) phenotype [15]. Further work re-enforced these findings. Li and coworkers [55] showed that, when administered after an active specific immunization, CTX augmented antitumor immunity by inducing TH1 commitment of antigen specific $CD4^+$ T cells. Zitvogel's group demonstrated that a single ip dose of CTX (100 mg/kg) could synergize with dendritic cell-derived exosome (Dex)-based therapy [56]. CTX could enhance tumor-induced T cell responses through inhibition of regulatory T cell (Treg) function leading to potent induction of specific tumor antigen $CD8^+$ T cells after Dex vaccination [56]. The clinical efficacy of this combination strategy (CTX + Dex) is currently under investigation in a phase II clinical trial in Gustave Roussy and Curie Institutes [12].

Cyclophosphamide and TH17

A new subset of T helper cells has recently emerged as a lineage distinct from TH1 or TH2 subsets, namely TH17 cells. Since their first description by Aarvak et al. in 1999, the regulatory pathways dictating TH17 differentiation and functions have been clarified. TH17 cells are defined by their secretion pattern, i.e., their production of IL-17A and IL-17F, IL-21 [57], IL-22 [58] regulated by the expression of two transcription factors, RAR-related orphan nuclear receptor (ROR), RORalpha, and RORgammat [59, 60]. TH17 cells play a key role in the pathogeny of autoimmune and inflammatory disorders [61–63] while exhibiting a protective function against certain types of bacterial infections. The precise function of TH17 during tumor immunosurveillance remains controversial [64–67], owing to the close plasticity and interconnections between TH17 and regulatory T cells in their differentiation pathways [68–70]. Accumulating data highlighted the presence of TH17 in tumor-infiltrating lymphocytes (TIL) in cancer patients [71–73]. In a study involving 30 patients with newly diagnosed lung cancer bearing malignant pleural effusion (MPE) [74], the authors showed that there was a correlation between the frequencies of TH17 and TH1 cells in pleural effusions associated with high levels of the pro-TH17 cytokines, IL-6, and IL-1β. In this study, a higher TH17 cell number in MPE was a significant predictor of improved overall survival [74]. A recent work conducted by Maruyama et al. [75] in a cohort of 55 patients bearing gastric cancer, a balance between TH17 and Treg cells in tumor-infiltrating lymphocytes, was geared toward TH17 in early disease stages while shifting to Treg at later stages of development [75].

In line with these observations and knowing that CTX could enhance TH1 polarization while decreasing the pool of Treg (see below), we investigated the impact of CTX on TH17 differentiation in mice and humans [12]. Indeed, CTX exerted a dose-dependent effect on the expansion or differentiation of $CD4^+$ T producing IL-17A, in naïve and tumor-bearing C57BL/6 mice. Although observed at metronomic dosing (50 mg/kg), these IL-17-producing TH cells did not result from the conversion of Treg into TH17. These data were confirmed in advanced cancer patients treated with non-myeloablative and non-lymphodepleting doses of CTX (3 weeks oral treatment with 50 mg/day). The levels of IL-17 secretion by circulating peripheral blood mononuclear cells (PBMC) after T cell receptors (TCR) stimulation (anti-CD3/CD28 Ab cross-linking) were significantly enhanced by the 3 weeks therapy with CTX. After cell sorting, we identified $CD4^+$ T cells as the major source of IL-17 post-CTX therapy. In one patient's ascitis fluid, CTX could also promote IL-17 and IFNγ secretion by TIL at days 16 and 54 compared with day 0. Altogether, these findings highlight that low doses of CTX increase the pool of TH17 cells in cancer bearing hosts. Whether CTX-induced TH17 contributes to the antitumor efficacy of CTX remains unclear as well as the main mechanisms accounting for TH17 differentiation in this setting.

Cyclophosphamide and Treg

Progressive tumors can escape immune recognition and destruction by actively establishing an immune tolerance involving immunosuppressive T lymphocytes [76–78]. Several subsets of immunosuppressive $CD4^+$ and $CD8^+$ T cells have been identified [79]. Recently, naturally occurring

cells exhibiting immunosuppressive (regulatory) functions were also identified as $CD4^+CD25^+$ regulatory T lymphocytes, in both rodents [80, 81] and humans [82]. These cells contribute to the prevention of autoimmune disorders by controlling the activity of autoreactive T lymphocytes. Treg also contribute to cancer-induced immune tolerance [83, 84]. Several studies performed on patients with various carcinomas reported that $CD4^+CD25^+$ Treg cells accumulated in the peripheral blood and tumor microenvironment [85–87].

Based on these assumptions, therapeutic depletion of these cells was harnessed to improve responses to cancer immunotherapy [88–91]. Ghiringhelli and coworkers [13] pioneered the field and found that among all the chemotherapeutic drugs currently used in the oncological armamentarium, CTX was the most efficient in ablating Treg in rodents. A single injection of low-dose CTX (30 mg/kg) strongly depleted $CD4^+CD25^+$ Treg cells by day 7 after treatment in rats. Although transient, this Treg depletion allowed cancer-specific immunotherapy to promote potent T cell and dependent antitumor effects. Later on, Ghiringhelli's group [92] showed that CTX not only depleted Treg in the blood and lymphoid organs of tumor-bearing animals but also decreased the number of Treg infiltrating tumor beds. The CTX-prodrug mafosfamide could indeed selectively induce cell death of FoxP3 expressing T cells both in vitro and in vivo. Indeed, Treg express Foxp3, a transcription factor, involved in their immunosuppressive effect [93] that is associated with an increased expression of proapoptotic molecules [94], which could contribute to their higher sensitivity to low-dose CTX [95].

Moreover, since Treg control NK-mediated antitumor immunity [96, 97], CTX-induced profound and selective reduction of circulating Treg restores innate killing activities both in mice [98] and in human [99]. Hirschhorn-Cymerman and Perales [100] originally proposed to combine CTX together with OX86 (an agonistic antibody targeting the costimulatory receptor OX40) for treating poorly immunogenic B16 melanoma tumors. This novel design of chemo-immunotherapy induced a profound Treg depletion accompanied by an influx of effector $CD8^+$ T cells leading to a favorable Teffector/Treg cell ratio, culminating in tumor regression. However, paradoxically, peripheral "bona fide" Treg cells greatly expanded. This phenomenon was the consequence of high-dose (250 mg/kg) CTX-induced lymphopenia. As recovery takes place and T cells undergo homeostatic proliferation, Treg proliferate more vigorously than other T cell subsets. In a microenvironment in which homeostatic proliferation is driven by self-MHC–peptide complexes [101], there are greater MHC–TCR interactions for Treg (whose repertoire is skewed toward self [102]) than for effector T cells. Interestingly, Lutsiak and coworkers [103] have shown that a single ip dose of CTX (100 mg/kg) has a direct inhibitory action on $CD4^+CD25^+$ Treg cells impairing their functionality in addition to decreasing cell numbers. CTX strictly affects the expression levels of GITR and FoxP3 which are involved in the suppressive activity of Treg, thus silencing their functionality. Recently, Nakahara et al. [21] proposed an indirect instead of a direct suppressive effect of CTX onto Treg. In this study, Treg expanded in vitro using $CD8\alpha^+$ DC from CTX-treated mice showed a greatly impaired suppressor potential on both $CD4^+$ and $CD8^+$ allogeneic T cell proliferation. Conversely, Treg expanded using $CD8\alpha^+$ DC (not exposed to CTX) was significantly more effective. This model subserves the hypothesis whereby resident $CD8\alpha^+$ DC represent a main component of peripheral tolerance through productive dialogues with Treg. Thus, CTX-induced depletion of the $CD8\alpha^+$ resident DC subset may shape immunological outcomes shifting host microenvironment from tolerogenicity to immunogenicity. Interestingly, the effects of CTX on Treg are transient and resolve generally within 10 days, reducing the theoretical possibility of promoting autoimmune disorders. Despite the assumption, well established in animal models, that CTX impairs both Treg frequency and activity, some reports contradict these findings, yet corroborating the antitumor effects of the chemoimmunotherapy regimen [47]. Therefore, it is of note that the Teffector/Treg ratio in tumor locations may be a better surrogate marker of efficacy of CTX than the baseline percentages of circulating Treg in advanced patients.

Cyclophosphamide and BCG: TRAIL-dependent effects

Tumor stroma is enriched by bone marrow-derived myeloid cells, including tumor-infiltrating DC (TIDC) or macrophages identified in virtually all human malignancies [104–110] and experimental tumor models [111–115]. The immunological functions of TIDC remain poorly understood [116]. The ability of DC to foster antitumoral immunity by triggering tumor cell death is an emerging concept [117, 118]. Tumor-infiltrating cells with natural killer (NK) functions and DC markers and functions, so-called IFN-producing killer DC, were demonstrated to kill tumor cells through a TNF-related apoptosis-inducing ligand (TRAIL)-dependent mechanism and control tumor growth [119–122]. In contrast, most reports documented the defective dialogue between DC and tumor cells or effector T lymphocytes in the microenvironment of malignancies [112, 123–125]. Ghiringhelli's group emphasized the efficacy of a combination of low-dose CTX and *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) in restoring the ability of DC to mediate an efficient antitumor immune response [92]. Working on colon carcinoma in rodents, they showed that the CTX–BCG combinatorial synergy resulted in a curative switch between pro-tolerogenic TIDC into T cell stimulating APC and TRAIL-expressing

Table 1 Chemoimmunotherapy combination with CTX in animal models

Dose and route	Synergy	Tumor model	Biological effect	Ref.
30 mg/kg ip (rats) 100 mg/kg ip (mice)	BCG rats: 8×10^5 CFU it 7 and 14 days post-CTX mice: 8×10^4 CFU it 2 and 7 days post-CTX	CT26 mouse colon carcinoma; PROb rat colon carcinoma	Curative switch between pro-tolerogenic TIDC into TRAIL-expressing killing DC and T cell stimulating APC resulting in complete tumor regression	[92]
83 mg/kg ip	Adoptive immune spleen cell transfer (10^8 cells iv) 5 h post-CTX	3Cl-8FLC; Eb lymphoma; ESb lymphoma; p11-R-Eb lymphoma	Lymphodepletion followed by cytokine-induced homeostatic proliferation, activation and trafficking of different lymphocyte pools. Cure rate: 100% (3Cl-8 FLC; Eb); 50% (p11-R-Eb); 37% (ESb)	[14]
100 mg/kg iv	TIL (2×10^7 cells ip) plus IL-2 (2×10^4 IU ip) 6 h post-CTX	MC-38 colon adenocarcinoma; MCA-105 sarcoma	Potentiation of transferred TIL; 75% long-term cure and resistance to subsequent tumor challenge	[144]
100 mg/kg ip	GM-CSF-secreting, HER-2 neu-expressing whole-cell vaccine (3×10^6 cells sc) 1 day post-CTX	Spontaneous neu-expressing breast tumor	TH1 polarization leading to Ag-specific antitumor immune response	[145]
100 mg/kg ip	HER-2 neu-expressing whole-cell vaccine (3×10^6 cells sc) 1 day post-CTX and DX (5 mg/kg iv) 1 week apart	Spontaneous neu-expressing breast tumor	Depletion of Treg cells favoring the vaccine-induced antitumor response; 10–30% cure rate	[146]
100 mg/kg ip	gp100-loaded exosomes (10 µg) plus ODN-CpG (20 µg) injected in the footpad 12 days post-CTX	B16 melanoma	Suppression of Treg function with markedly enhanced secondary CTL responses induced by exosome vaccination; 30% cure rate	[56]
100 mg/kg ip	Adoptive immune spleen cell transfer (5×10^7 cells iv) 5 h post-CTX	3Cl-8 FLC; RBL-5 leukemia	Lymphocyte homeostatic proliferation, activation, homing into secondary lymphoid organs and migration into tumor bed; 100% tumor regression with no tumor recurrence	[16]
100 mg/kg ip	DC101(mAb α-VEGF-R2 0.8 mg ip) and GM-CSF-secreting, HER-2 neu-expressing whole-cell vaccine (3×10^6 cells sc) 1 day post-CTX, plus DX (5 mg/kg iv) 1 week apart	Spontaneous neu-expressing breast tumor	Depletion of Treg cells favoring the combined DC101 plus neu-vaccination-mediated increased tumor-specific $CD8^+$ T cell activity; 40% survival rate	[142]
100 mg/kg ip	Anti-CD25 PC61 mAb (50 µg ip) injected with CTX 1 day before whole-cell vaccine (Panc02 and B78H1-GM cells; 4×10^7 cells sc)	Mesothelin-expressing pancreatic tumor model Panc02	Depletion of Treg cells responsible for the increased tumor-specific $CD8^+$ T cell expansion correlating with enhanced vaccine efficacy	[147]
100 mg/kg ip	Type I IFNs (10^5 IU pt) daily for 4 days starting from day 1 post-CTX	EG.7 thymoma; RBL-5 leukemia	60% of tumor regression with long-term survival and resistance to subsequent tumor challenge	[22]
100 mg/kg ip	Dimethyl amiloride (1 µmol/kg ip) daily	CT26 colon carcinoma; EL4 thymoma; TS/A mammary adenocarcinoma	Tumor-derived exosome depletion leading to potent synergistic antitumor efficacy	[148]
150 mg/kg iv	Adoptive T cell transfer (2×10^8 cells iv) 1 h post-CTX	L5178Y lymphoma	Elimination of tumor-induced suppressor T cells resulting in complete tumor regression and in long-term survival	[7]
150 mg/kg ip for 3 days	Syngeneic BM-derived DC (10^6 cells it) injected on days 12, 13, 14 and 18 post-CTX	CT26 colon carcinoma	Complete tumor regression with persistent systemic antitumor immune memory	[149]
Iterative 150 mg/kg vs. metronomic 175 mg/kg ip	Recombinant plasmid DNA-*mel3* im plus recombinant modified vaccinia virus Ankara encoding the *mel3* polyepitope construct (10^6 plaque-forming units iv)	B16 melanoma	Synergistic antitumor response when immunotherapy was combined with metronomic delivery of CTX. The treatment caused a reduction in CTL numbers while selectively sparing CTL with memory capacity	[150]
200 mg/kg	Thymosin α1 (200 mg/kg) for 4 days plus a single injection of IFNα/β (3×10^4 IU) starting 2 days post-CTX	3LL Lewis lung carcinoma	Stimulation of NK activity against autologous 3LL tumor cells leading to long-term survival	[151]
200 mg/kg ip	IM (50 mg/kg/day ip) and IFNα (1,000 UI/day) 5 times/week for 1 or 2 weeks	*bcr-abl*-transformed B210 and 12B1 leukemic cells	100% cure rate. No biological effect is described	[152]

Table 1 (continued)

Dose and route	Synergy	Tumor model	Biological effect	Ref.
200 mg/kg ip	Adoptive transfer of naive pmel-1 Ly5.1 cells (1×10^6 cells iv) 1 day post-CTX and gp100 (25–33) melanoma peptide (100 µg sc) plus poly(I:C) (200 µg sc) at day 2 and 12 post-CTX	B16 melanoma	DC expansion and activation with increased inflammatory cytokine production and effective antitumor pmel-1 $CD8^{(+)}$ T cell response; 100% cure rate	[19]
250 mg/kg ip	OX86 (anti-OX-40 Ab, 0.5 mg ip) 1 day post-CTX	B16 melanoma	Treg depletion in tumor beds accompanied by $CD8^+$ T cell influx leading a favorable Teff/Treg cell ratio; 75% cure rate	[153]

HER-2 human epidermal growth factor receptor 2, *DEX* DC-derived exosomes, *VEGFR* vascular endothelial growth factor, *TIDC* tumor-infiltrating dendritic cells, *ip* intraperitoneally, *it* intratumorally, *iv* intravenously, *sc* subcutaneously, *pt* peritumorally, *im* intramuscularly

TIDC capable of killing. BCG could upregulate TRAIL expression on $CD11b^+$ DC through a TLR2, TLR4, and TLR9 signaling. Since Treg compromised the BCG-induced upregulation of TRAIL on DC, blocking TRAIL-dependent cytotoxicity, the coinjection of CTX leading to Treg depletion together with BCG restored the immunogenicity of the tumor microenvironment, resulting in efficient antitumor responses. They further confirmed their results in human monocyte-derived DC that expressed TRAIL after in vitro stimulation with BCG, except in the presence of human Treg. Altogether, Treg can control TLR-dependent TRAIL expression on TIDC, a phenomenon that can be entailed by combining CTX and BCG. Accordingly, Van der Most and coworkers [126] have proposed a CTX-induced TRAIL-dependent tumor apoptosis. Therapy of mesothelioma with CTX resulted in the killing of the majority of tumor cells and sensitized the bystander chemoresistant counterparts to a TRAIL-mediated lysis mediated by CTX-activated T cells. An intriguing possibility is that TRAIL-mediated tumor cell killing contributes to immunogenic cell death [127]. This model offers a new explanation for the paradoxical effect of lymphodepleting chemotherapy whereby tumor growth is controlled by qualitatively different lymphocytic infiltrates. Targeting TRAIL receptors (extrinsic cell death) might synergize with other cell death pathways such as those mediated through perforin (intrinsic cell death) or through angiogenesis (rIFN-γ, anti-VEGF Ab).

Cyclophosphamide as an adjuvant in cancer: preclinical and clinical studies

Immunotherapy is an attractive and potentially effective alternative strategy of cancer treatment, based on its specificity and limited associated toxicity [128]. While for decades therapeutic vaccination was not indicated as a standard anticancer therapy [129], the recent FDA approval of a DC vaccine [130] and the success of overlapping long peptides [131] and anti-CTLA4 antibodies [132] have fueled novel research programs aimed at combining these immunological components together with standard chemotherapy.

Due to its multifaceted immunological properties, CTX combined to immunotherapy may affect antigen cross-presentation [126], induce a "cytokine storm" [16], reduce the number of intratumor regulatory T cells [99], and activate homeostatic lymphoid proliferation [14, 133]. Therefore, several preclinical studies combined low or high dosing of CTX with various strategies of immunotherapy such as the adoptive transfer of T lymphocytes, tumor peptides, GM-CSF engineered dying tumor cells, DC, exosomes [56], TLR agonists, type I IFN, imatinib mesylate [134], or anti-OX40 Ab (for a review, see Table 1).

Based on these premises of efficacy, numerous investigators used metronomic CTX alone or together with anticancer vaccines in clinical trials and monitored immunological parameters (for a review, see Table 2). The pioneering work of Mastrangelo and colleagues demonstrated that CTX could boost immune responses in patients [135]. In the 1980s, they combined CTX (300 mg/m^2 iv) to autologous melanoma cell vaccines (administered 3 days later) in advanced melanoma patients. This 28-day regimen significantly triggered delayed-type hypersensitivity (DTH) responses to the vaccine in 89% of patients [136] and selectively impaired $CD45^+$ T cell-mediated suppressor activity [137], eventually associated with 12% objective responses.

Ghiringhelli et al. have reported that metronomic CTX (50 mg/day 1 week on 1 week off for 1 month) is a valuable treatment to reset T and NK cell responses blunted in end stage cancer patients [99]. This metronomic regimen led to a selective reduction of circulating Treg, restoring innate and acquired TCR-driven T cell responses in nine patients. However, at a higher dosing of CTX (twice the dose), a systemic lymphodepletion occurred.

Jaffee et al. elaborated a protocol combining GM-CSF engineered tumor vaccines together with metronomic CTX

Table 2 Clinical trials assaying CTX as a cancer adjuvant

Dose and route	Malignancy	Biological outcome	Clinical outcome	Ref.
CTX 50 mg per os, twice/day 1 week on and 1 week off	End stage colon; renal; gastric; rectal; ovarian cancers, mesothelioma; melanoma; sarcoma	Selective and profound reduction of Treg cell number and functions and restoration of TCR-driven T cell proliferation and NK cell cytotoxicity	44.4% disease stabilization	[99]
CTX (50 mg/day) per os plus IM (400 mg/day)	Radiation-induced midbrain fibrosarcoma	No biological effects are described	Progression-free survival of 6 months	[154]
CTX 50 mg per os with prednisolone (10 mg per os daily)	Metastatic hormone-refractory prostate cancer	No biological effects are described	Decrease of PSA ≥50%, 26% of patients ≥30%, 48% of patients	[155]
CTX 60 mg/kg iv for 2 days and fludarabine (25 mg/m^2 iv) for 5 days plus tumor-reactive lymphocytes and IL-2 (7.2×10^4 IU/kg iv)	Refractory metastatic melanoma	Long-term persistence of transferred tumor Ag-reactive lymphocytes	51% overall response rate (8.6% complete and 42.9% partial)[c] up-raising to 70% when 12 Gy TBI was added[d]	[156, 157]
CTX 200 mg/m^2 iv 1 day before allogeneic, HER2-positive, GM-CSF-secreting tumor vaccine (5×10^8 cells) and 8 days before DX (35 mg/m^2)	Metastatic breast cancer	Induction of HER2-specific humoral immunity	This phase I study showed that immunotherapy with allogeneic, HER2-positive, GM-CSF-secreting tumor vaccine is safe	[139]
CTX 200 mg/m^2 iv bolus 7 days before GM2 (200 μg id) mixed to BCG (10^7 viable units)	Metastatic melanoma	Induction of IgM anti-GM2-Ab responses in most patients correlating with longer disease-free survival	23% increase in disease-free interval; 14% increase in overall survival	[158]
CTX 250 mg/m^2 iv 1 day before GM-CSF-secreting pancreatic cell lines CG8020 and CG2505 (5×10^8 cells, 4–8 id injections)	Metastatic pancreatic cancer	CD8$^+$ T cell responses to HLA class I-restricted mesothelin epitopes	40% disease stabilization with a median survival value of 4.3 months compared to 2.3 months in patients receiving vaccine alone	[138]
300 mg/m^2 iv bolus	Metastatic melanoma; colorectal/breast/lung adenocarcinoma	Augmented antibody response and DTH responses to KLH (100% of patients)	No objective responses	[159]
300 mg/m^2 iv bolus 3 days before autologous MCV (10–25×10^6 cells id)	Metastatic melanoma	Augmented DTH responses to autologous MCV (in 89% cases)[a], selective impairment of CD45$^+$ T cell-mediated suppressor activity of PBL[b]	9.1% (3/33) complete responses[a,e] 3% (1/33) partial responses[e]	[136, 137, 160]
CTX 300 mg/m^2 iv bolus 3 days before MCV (10–25×10^6 cells id) mixed to BCG	Metastatic melanoma	DTH to autologous melanoma cell-associated Ag and metastasis lymphocytic infiltration in regressing metastases	10% complete responses; 2.5% partial responses; 15% delayed responses	[161]
CTX 300, 150, or 75 mg/m^2 iv bolus 3 days before MCV	Metastatic melanoma	Dose-dependent reduction of peripheral blood CD8$^+$CD11b$^+$ suppressor cell activity, correlating with clinical responses	Disease stabilization: MCV + CTX (300 mg/m^2)—66.7%; MCV + CTX (150 mg/m^2)—60%; MCV + CTX (75 mg/m^2)—50%	[162]
300 mg/m^2 iv bolus 3 days before Melacine plus Detox-PC(2×10^7 cell equivalents once a week, 5 weeks) and IFN (5×10^6 IU/m^3, 3 times/week 1 month post-CTX)	Metastatic melanoma	No biological effects are described	10.2% response rate (5.1% CR, 5.1% PR) 64% disease stabilization	[163]
300 mg/m^2 iv infused in 2 h 3/4 days before tumor lysate- and KLH-loaded moDC iv or id	Metastatic renal cell carcinoma	KLH specific immune responses only in patients with tumor regression; Ag-independent PBMC proliferation in the remaining patients	9.1% tumor regression; 13.6% disease stabilization	[164]
DTIC 800 mg/m^2 iv bolus 1 day before Melan-A and gp 100 peptides (250 μg each id) plus IFNα (3MU sc)	Melanoma (resected, disease-free patients)	Enhancement of long-lasting memory CD8$^+$ T cell response and of TCR repertoire diversity accompanied by high avidity and tumor reactivity	60% (3/5) disease-free patients	[140, 141]
1,000 mg/m^2 iv bolus	Metastatic melanoma; colorectal carcinoma	Enhanced DTH responses to KLH (100% of patients) and to 1-chloro-2,4-dinitrobenzene	13.6% partial responses	[135]

Table 2 (continued)

Dose and route	Malignancy	Biological outcome	Clinical outcome	Ref.
		(27% of patients)		
1,000 mg/m^2 iv bolus	Metastatic malignant melanoma; tissue sarcoma, colon/breast/ovarian/ prostate/pancreatic carcinoma	Impairment of T cell-mediated suppressor activity of PBL	5.2% partial responses	[165]

KLH keyhole limpet hemocyanin, *PBL* peripheral blood lymphocytes, *MCV* melanoma cell vaccine, *CR* complete response, *PR* partial response, *TBI* total body irradiation, *IM* imatinib mesylate, *id* intradermally

[a] [136]

[b] [137]

[c] [156]

[d] [157]

[e] [160]

(250 mg/m2 iv 1 day before the vaccine). First, the investigators conducted a study in advanced pancreatic cancer patients and reported not only safety of the combination chemoimmunotherapy regimen but also enhanced anti-mesothelin CD8$^+$ T cell responses and median overall survival in the cohort receiving CTX and vaccine compared with the one receiving vaccine alone [138]. Next, they conducted a study aimed at assessing the appropriate dosing of CTX to obtain synergistic immunological effects with vaccines and combined HER2-expressing GM-CSF secreting tumor vaccine together with various dosages of CTX and doxorubicin (DX). While HER2-specific DTH

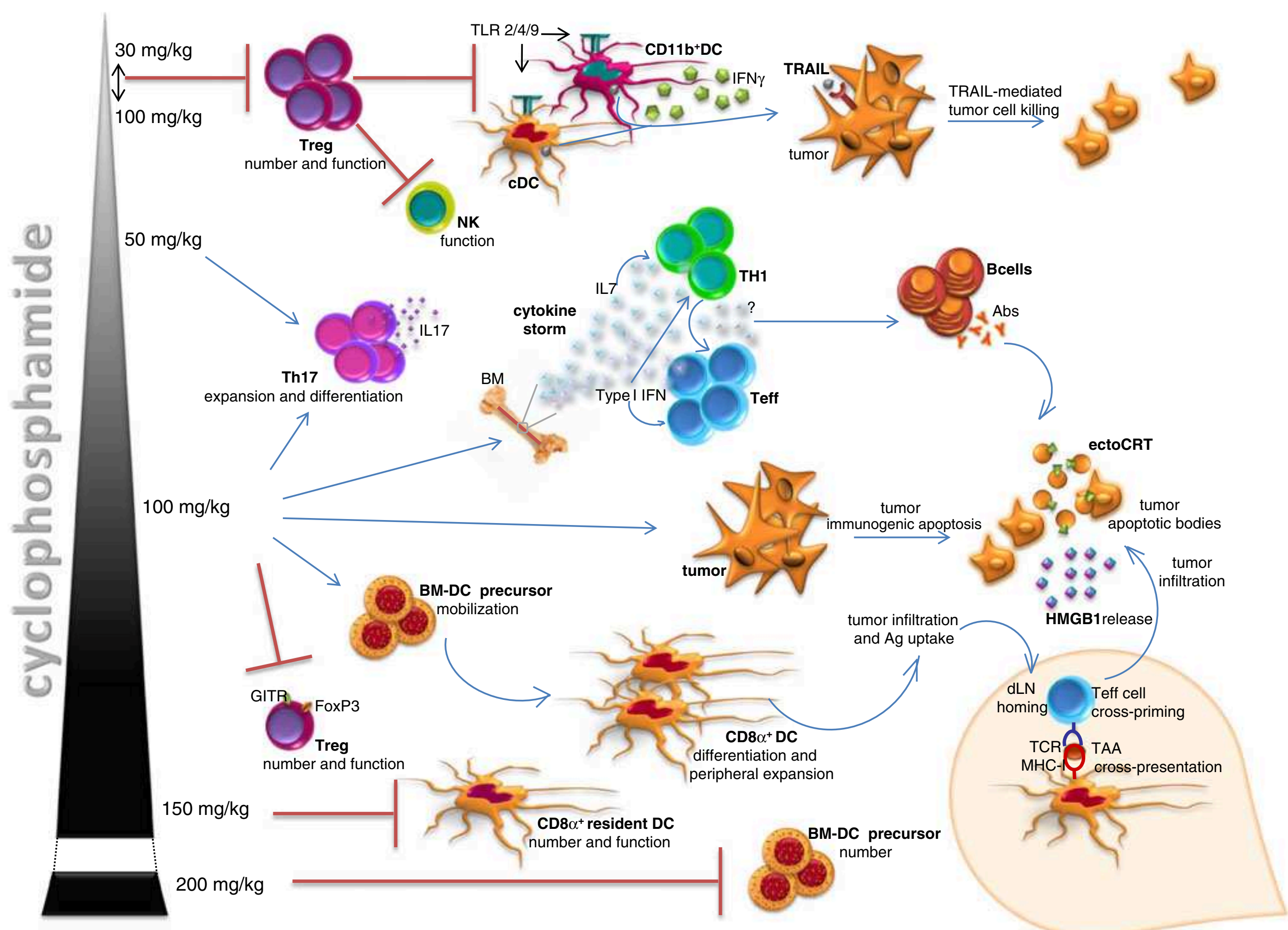

Fig. 1 Example of a chemotherapy exhibiting potent immunomodulatory effects

developed regardless of chemotherapy, the highest antibody titers against HER2 were obtained with CTX at 200 and DX at 35 mg/m^2. At higher dosing of CTX, humoral immune responses were suppressed [139].

Proietti's group recently performed a pilot clinical study aimed at evaluating the safety and the antitumor immune responses of the combination of another alkylating agent, dacarbazine (DTIC) together with a peptide-based vaccine in stages II–IV disease-free melanoma patients [140, 141]. Preclinical data indicated that DTIC shared with CTX its immunomodulatory properties in mouse tumor models (unpublished observations). Patients received an infusion of DTIC 1 day before immunization with peptide (Melan A/MART1) based cancer vaccine for six cycles (every 21 day). The investigators compared the diversity of the T cell repertoire against the vaccine peptide and the avidity of Melan A-specific T cell responses prior to and following the combination therapy. This iterative therapy revealed that DTIC administration prior to peptide vaccination could result in an improved cellular immune response to the vaccine peptide and in an early upregulation of immune response-related genes. A remarkable ex vivo expansion of peptide specific CD8$^+$ T cells displaying a long-lasting effector memory phenotype and ability to specifically lyse human leukocyte antigen (HLA)-A21/Melan A1 tumor cell lines was demonstrated only in immunized patients pre-treated with DTIC. They observed a broader usage of Mart-1 specific TCR post-therapy in patients receiving DTIC. Notably, analysis of PBMC gene expression profiles revealed an increased expression of immunoregulatory factors 1 day after chemotherapy. DTIC administration before peptide vaccination was safe, well tolerated, and able to induce a long-lasting enhancement of memory CD8$^+$ T cell responses to cancer vaccines.

Future prospects

CTX represents the oldest and most extensively studied example of a chemotherapy exhibiting potent immunomodulatory effects (Fig. 1). However, several hurdles have discouraged many investigators to exploit this strategy of immunomodulation. So far, the scientific grounds accounting for the so-called specificity of the CTX-mediated immunomodulation were not clear. Indeed, DX or DTIC at low dosing can also mediate immunostimulation. Moreover, the precise dose, route, and scheduling for the CTX-based chemoimmunotherapies remained unclear for decades. Importantly, the frontiers between the antiangiogenic effects and the immunomodulatory effects of CTX remain sketchy given that TH1 and IFNγ delivered by activated T lymphocytes might prevent and/or destroy tumor neovessels. The complexity comes from the fact that specific antiangiogenic compounds (targeting VEGFR2) might also promote antitumor effects through immune-dependent mechanisms [142].

In the whole arsenal of immunomodulatory agents, CTX remains an interesting compound, devoid of toxicity at low dosing and affordable. Nevertheless, CTX will have to be compared or combined with new reagents (such as anti-CTLA4 Ab, anti-PD-1 or PDL-1 Ab, agonistic anti-CD40 or 4-1BBL Ab….) and established drugs [143] that together with efficient vaccines may offer valuable cancer adjuvants in the future.

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Prerequisites for the Antitumor Vaccine-Like Effect of Chemotherapy and Radiotherapy

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Abstract: For a long time, anticancer therapies were believed to work (and hence convey a therapeutic benefit) either by killing cancer cells or by inducing a permanent arrest in their cell cycle (senescence). In both scenarios, the efficacy of anticancer regimens was thought to depend on cancer cell–intrinsic features only. More recently, the importance of the tumor microenvironment (including stromal and immune cells) has been recognized, along with the development of therapies that function by modulating tumor cell–extrinsic pathways. In particular, it has been shown that some chemotherapeutic and radiotherapeutic regimens trigger cancer cell death while stimulating an active immune response against the tumor. Such an immunogenic cell death relies on the coordinated emission of specific signals from dying cancer cells and their perception by the host immune system. The resulting tumor-specific immune response is critical for the eradication of tumor cells that may survive therapy. In this review, we discuss the molecular mechanisms that underlie the vaccine-like effects of some chemotherapeutic and radiotherapeutic regimens, with particular attention to the signaling pathways and genetic elements that constitute the prerequisites for immunogenic anticancer therapy.

Key Words: Calreticulin, HMGB1, NLRP3, TLR4, ATP, dendritic cells

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IMMUNOGENIC TUMOR CELL DEATH

Although the armamentarium of anticancer therapies is being constantly ameliorated, the number of people succumbing to cancer has been predicted to drastically rise in the future (www.who.int/cancer). This trend basically reflects the facts that efficient therapies for some prominent neoplasms such as lung cancer (which now is the leading cause of cancer-related deaths worldwide) are still missing and that current anticancer regimens are often associated with the insurgence of resistance and therapeutic failure.[1] To counteract this dreadful tendency, further insights into the molecular mechanisms underlying the resistance of cancer cells to conventional therapy and into the signaling pathways that govern the host-tumor crosstalk are urgently awaited. This type of information will allow not only for the refinement of the current therapeutic arsenal, but also for a better stratification of cancer patients and the development of personalized anticancer therapies.

The current clinical approach to cancer most frequently involves surgery (whenever possible) alone or in association with a single-agent or combinatorial treatment based on chemotherapy or radiotherapy. Intriguingly, some cancers can be cured by conventional regimen (such as breast, colon, testicular, prostate, head and neck cancers, Hodgkin and follicular lymphoma, etc.), whereas others still remain a major medical challenge (such as lung and pancreatic cancers), suggesting that the intrinsic properties of the tumor and/or the specificity of the cytotoxic drugs matter. Minimal residual disease or incomplete eradication of tumor (stem) cells associated with the arousal of chemoresistant and/or radioresistant metastases[1] questioned the bases of our current reasoning. Thus, to improve the clinical outcome of anticancer therapies, it is of the utmost importance to understand how therapy-resistant tumor cells can be efficiently targeted and how therapeutic failure can be predicted.

For a long time, the field of clinical oncology was dominated by the notion that efficient anticancer therapies would work exclusively on tumor cells, either by inducing their apoptotic demise (cytotoxicity) and immunologically silent clearance or by permanently arresting their cell cycle progression (cytostasis). Moreover, several anticancer compounds were known to induce different degrees of immunosuppression, reinforcing the belief that the host immune system plays no role in the fight against transformed cells. Even the official guidelines formulated in 1975 by the National Institutes of Health recommended that the efficacy of novel anticancer strategies should be evaluated on human cancers xenotransplanted in immunodeficient mice.[2] More recently, it has been shown that (i) cancer cells engage in a strict crosstalk with their microenvironment (the tumor stroma, including fibroblasts and endothelial and immune cells such as macrophages) and that this interaction can be specifically targeted to induce tumor regression[3]; that (ii) highly efficient anticancer regimens can kill tumor cells through nonapoptotic cell death subroutines[4]; and that (iii) apoptosis can also occur in an immunogenic fashion, leading to the elicitation of an anticancer immune response.[5]

Such an immunogenic cell death (ICD) involves the transfer of tumor-derived antigens to immune cells that stimulate a tumor-specific immune response. This is critical for the eradication of residual cancer (stem) cells as it operates irrespective of their resistance to therapy.[2] Experiments in suitable animal

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models demonstrated that the subcutaneous inoculation of dying cancer cells (in the absence of any adjuvant) into syngenic immunocompetent mice exerts a vaccine-like effect, thereby protecting animals against subsequent challenges with live tumor cells of the same type.[5,6] Moreover, in preclinical tests, some anticancer treatments (such as doxorubicin, oxaliplatin, cyclophosphamide, and ionizing radiation) turned out to be much more efficient in immunocompetent than in immunocompromised settings.[6–8] This effect has been shown to rely on different components of the host immune system, as it is lost in mice lacking T or dendritic cells (DCs), interferon γ (IFN-γ) or its receptors, interleukin (IL) 17 or its receptor, and NLR family, pyrin domain containing 3 (NLRP3, a component of the innate immune system).[5,6,9–11] We surmise that the cognate immune response induced by immunogenic anticancer regimens is dictated by (i) the emission of immunogenic factors from dying tumor cells and (ii) the perception of these signals by DCs, which engulf, process, and present antigens to stimulate IFN-γ–producing CD8+ (Tc1) cells.

We discuss the latest developments in the field of ICD and their relevance for anticancer chemotherapy and radiotherapy.

CELL DEATH–ASSOCIATED MOLECULAR PATTERNS

The molecular pathways that are responsible for the elicitation of an antitumor immune response upon ICD involve the emission of specific cell death–associated molecular patterns (CDAMPs) that—in a correct spatiotemporal and temporal appearance—bear the ability to convert nonimmunogenic corpse removal into an immunogenic reaction. Obviously, such a conversion also relies on the correct perception of CDAMPs by dedicated components of the host immune system.

Thus, antigens from cancer cells succumbing to ICD inducers such as anthracyclines and ionizing radiations are efficiently taken up and processed by DCs, which in turn cross-prime naive T cells and drive the development of a tumor-specific immune response.[12] The interaction between DCs and dying cancer cells is controlled by the emission and/or release from the latter of so-called "eat me" signals and "don't eat me" signals, i.e., membrane-bound or soluble molecules that stimulate or inhibit phagocytosis, respectively.[13] The systematic analysis of surface proteome alterations in anthracycline-treated tumor cells revealed that ICD is associated with the ectopic coexposure of the endoplasmic reticulum (ER) chaperones calreticulin (CRT) and ERp57.[6,14] Subsequent studies demonstrated that ecto-CRT functions as an eat-me signal for DCs, thereby facilitating cell–death-mediated antigen uptake, and is an absolute requirement for the immunogenicity of dying tumor cells.[6]

The coexposure of CRT and ERp57 reportedly ensues the induction of an ER stress response that is associated with massive ultrastructural alterations of this organelle and depends on the activation of (at least) 3 signaling modules.[15,16] First, the ER-resident protein kinase R–like ER kinase gets activated and couples ER stress signals to translation inhibition by phosphorylating the eukaryotic initiation factor 2α (eIF2α). Accordingly, the disruption of the eIF2α phosphatase complex PP1/GADD34 by small peptide inhibitors, resulting in increased eIF2α, suffices to trigger CRT exposure in cancer cells.[17] Second, an apoptotic module that involves the mitochondrion-permeabilizing proteins BAX and BAK (which also work at the interface between the ER and mitochondria to regulate calcium fluxes)[18,19] caspase 8 and its substrate BAP31—an ER sessile protein implicated in the lethal response to ER stress—is activated. Thus, the pan-caspase inhibitor Z-VAD-fmk, as well as genetic interventions whereby BAX, BAK, and/or caspase 8 are removed or depleted, blocks CRT exposure and abolishes the tumor-vaccinating effect of cells undergoing ICD.[6,15]

Third, approximately 5% to 10% of the endogenous CRT pool is exposed together with ERp57 at the surface of dying cells via SNAP and NSF attachment receptor (SNARE)–dependent exocytosis. This occurs well before plasma membrane permeabilization (which occurs as the final step of apoptosis) and also precedes the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Phosphatidylserine is the prototypic eat-me signal of apoptotic cells (although it has been implicated also in nonapoptotic cell death),[20,21] and the kinetics of its exposure might affect the switch between the silent removal of dying cells by macrophages and the initiation of a cognate immune response by DCs.[16] The receptor that is responsible for antigen uptake by DCs upon CRT binding remains to be determined. Possible candidates include the major CRT receptor CD91 as well as other CRT-interacting proteins such as scavenger receptor A, scavenger receptor expressed on endothelial cell I,[22] CD40 ligand, tumor necrosis factor–related apoptosis-inducing ligand (tumor necrosis factor–related apoptosis inducing ligand), or CD95/FAS ligand.[23] The CRT-driven uptake of tumor antigens by DCs is per se insufficient to elicit an antitumor immune response as internalized antigens must be processed and re-exposed for the cross-priming of CD4+ and CD8+ T lymphocytes. This implies that other signaling pathways are involved in ICD.

A systematic study of the response to CDAMPs of distinct Toll-like receptors (TLRs) on naive T cells revealed that TLR4 is both required and sufficient for efficient antigen presentation by DCs.[24] Among other proteins, TLR4 binds the non–histone chromatin–binding nuclear protein high-mobility group box 1 (HMGB1), leading to the activation of the downstream effector myeloid differentiation primary response 88 (MYD88).[24] This inhibits the fusion between lysosomes and antigen-containing phagosomes, thus facilitating antigen processing and presentation to T cells. High-mobility group box 1 also stimulates the neosynthesis of pro–IL-1β[25] but per se does not serve as a DC maturation signal.[24] For a long time, HMGB1 has been thought to exert a proinflammatory function exclusively during necrosis,[26] but recent evidence indicates that it also gets released during the late stages of apoptosis.[27] The release of HMGB1 from tumor cells succumbing to ICD can be blocked by Z-VAD-fmk and hence depends on the activation of caspases.[24,27] This process manifests with a dual kinetics whereby HMGB1 first translocates from the nucleus to the cytoplasm and then, following the breakdown of the plasma membrane, gets released into the extracellular space.[8] Further insights into the molecular mechanisms that underlie HMBG1 release are missing. However, the addition of recombinant CRT or HMGB1 to dying cancer cells does not suffice to stimulate the presentation of tumor antigens by DCs,[28,29] implying that additional signals are required for the immunogenicity of cell death.

The vaccine-like effect of ICD relies on the elicitation of an IFN-γ–polarized T-cell response, which in turn requires the function of the NLRP3 inflammasome, a multiprotein caspase 1–activating complex[30] Caspase 1 activation is critical for activating an antitumor immune response as it catalyzes the proteolytic maturation of IL-1β.[31] One of the most abundant factors that activate the NLRP3 inflammasome is ATP,[32] and—at least in DCs—it does so by binding to the purinergic P2RX7 receptor on the cell surface.[10] ATP also constitutes a CDAMP, as it gets released during the final steps of cell death, possibly via

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voltage-gated hemichannels of the pannexin 1 or connexin type.[33] Accordingly, the depletion of intracellular or extracellular ATP in cells succumbing to ICD abolishes the development of an IFN-γ–polarized response, and P2RX7-deficient mice fail to mount an immune response against syngenic cancer cells succumbing to ICD.[10] Intriguingly, ATP also serves as a "find me" signal for the attraction of immune cells.[34] Altogether, these observations highlight the multifaceted and critical role of ATP for the vaccine-like effects of ICD inducers.

SPATIOTEMPORAL CODE

The spatially and temporally regulated emission of immunogenic factors from dying tumor cells accounts for the recruitment and activation of immune cells to tumor bed and governs the immune response to cancer cells undergoing ICD (Fig. 1). Thus, the stress conditions that cancer cells confront during chemotherapy and radiotherapy determine whether the subsequent wave of cell death will elicit an antitumor immune response or rather will remain immunologically silent. Normally, cells attempt to cope with stress by arresting normal activities and by activating a series of cytoprotective mechanisms that aim at reestablishing homeostasis. For instance, stressed cells normally arrest protein synthesis, activate DNA repair pathways, and up-regulate factors for the handling of unfolded proteins as well as antioxidant defenses. This is accompanied by alterations of the surface proteome that, in the case of ICD, account for the recognition by immune cells and by the emission of soluble mediators with chemotactic and antichemotactic properties. This is crucial for the "selection" and differentiation/maturation of engulfing cells, which in turn dictates the immunogenic or tolerogenic outcome of cell death.

In this sense, the exposure of the DC-specific eat-me signal CRT (well before that of PS) paralleled by the disclosure of other, hitherto uncharacterized "don't eat me" signals (such as CD47 [35–37]) facilitates the recognition and uptake of dying tumor cells by DCs rather than by macrophages. Other authors have described additional molecular components exposed by dying cells that should be recognized for engulfment by specific DC subsets (such as CLEC9A/DNGR1 and HSP70/90) to elicit adaptive immune responses.[29,38,39] However, additional stimuli released by dying tumor cells in the proximity of DCs are indispensable for the induction of a tumor-specific immune response. Thus, HMGB1 and ATP facilitate antigen processing/presentation and the release of IL-1β, which is necessary for IFN-γ–polarized T-cell responses. Based on these observations, the spatially restricted and temporally ordered appearance of CRT, HMGB1, and ATP might constitute a "key" that would precisely fit into a series of pattern recognition receptors expressed by DCs (the "lock") for the conversion of nonimmunogenic into ICD and for the elicitation of an anticancer immune response.[12]

THE PERCEPTION OF IMMUNOGENIC CELL DEATH BY THE IMMUNE SYSTEM

Oncogenesis is a multistep mechanism that also involves an escape from immunosurveillance; that is, often cancer cells that sustain tumor growth are poorly immunogenic (immunoselection) and/or they actively inhibit immune functions (immunosubversion).[40] In this context, there are (at least) 2 different pathways whereby the immune system can be recruited against tumors: via direct immunomodulatory therapies that relieve immunosuppression or indirectly upon the induction of ICD.

By shaping T-cell responses, DCs are the first-line decision makers of the innate immune system, and their role in immunogenic chemotherapy has been deeply investigated. Experiments in transgenic mice that express the diphtheria toxin receptor under the control of a DC-specific promoter (allowing for in vivo DC depletion)[9] revealed the essential role of DCs in the perception and decoding of "come and get me" signals

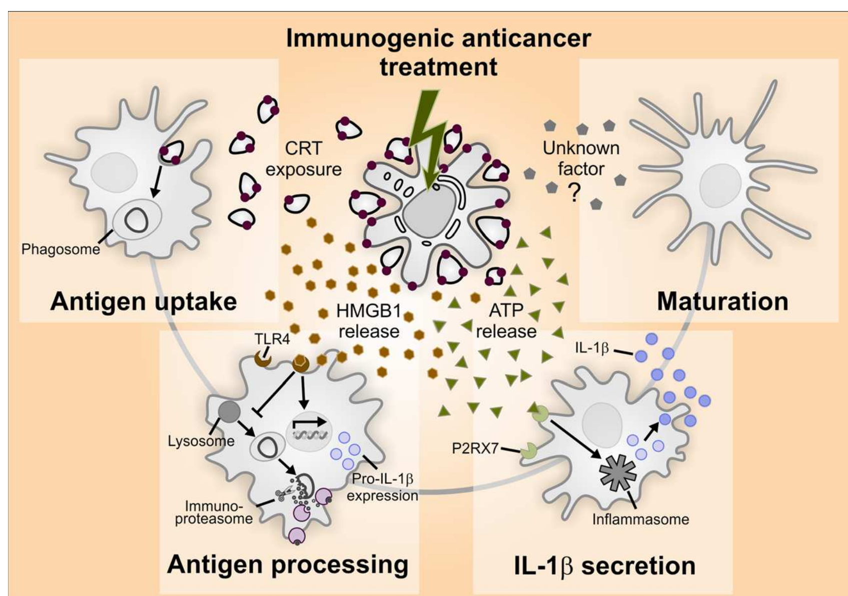

FIGURE 1. Immunogenic signals emitted by dying cells form a spatiotemporal code unlocking DCs to mount a potent immune response toward tumor cells. (i) Early exposure of ecto-CRT by dying tumor cells, which facilitates engulfment by DCs. (ii) HMGB1 released from dying cells binds to TLR4 on DCs, thus favoring antigen cross-presentation and up-regulating pro–IL-β (pro–IL-1β). (iii) ATP liberated from dying cells binds to the purinergic receptor P2RX7 on DCs, activates the NLRP3 inflammasome, and leads to the secretion of active IL-1β, which polarizes CD8+ T cells toward IFN-γ production. (iv) An additive DC maturation factor remains to be characterized.

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emitted by dying tumor cells during ICD.[24] Similarly, the in vivo depletion of CD8+ T cells with specific antibodies has been instrumental to highlight the critical role of this lymphocyte subset for the vaccine-like effect of chemotherapy and radiotherapy in a large panel of murine tumor models, including CT26 colon cancer, EL4 thymomas, TS/A mammary carcinomas, MCA205 fibrosarcomas, Glasgow osteosarcoma osteosarcomas,[11,24] and spontaneous methylcholanthrene-induced sarcomas.[24,41] In line with these observations, CD8+ T cells have been shown to mediate potent anticancer immune effects in clinical settings, for instance, in colorectal tumors, where immune infiltration might serve as a prognostic factor.[42] Moreover, it has recently been shown that a precise orchestration of the T-cell response is required for immune effectors to eradicate tumors.[43] In this context, the IL-1β–dependent activation of IL-17–secreting γ/δ T cells had to precede the infiltration of tumors by Tc1 lymphocytes for the efficacy of immunogenic chemotherapy in vivo.[43] Thus, a finely regulated crosstalk between components of the innate (DCs) and cognate (γ/δ and CD8+ T cells) immune system is required for cell death to be perceived as immunogenic, for the elicitation of an anticancer immune response, and for complete tumor eradication leading to therapeutic success (Fig. 2). However, how resident macrophages and/or adverse inflammatory monocytes, which contribute to the proangiogenic and protumoral microenvironment and dominate the scenario before chemotherapy, become "transformed" and/or "overruled" by a subset of antigen presenting cells capable of eliciting a protective anticancer responses in the context of ICD remains to be established.

IMMUNOGENIC ANTICANCER CHEMOTHERAPY

The current definition of immunogenic chemotherapy is based on the ability of a limited array of antineoplastic drugs to elicit ICD rather than the stereotypical, immunologically silent or even tolerogenic apoptotic pathway. A plethora of preclinical[44–51] and clinical[52] studies revealed that DCs can take up apoptotic tumor cells and cross-present the internalized antigens on major histocompatibility complex class I molecules to CD8+ T cells, thus eliciting a productive immune response.

These studies suggest that the immunogenic outcome of cell death is influenced (among other factors) by the nature of the tumor cell as well as by the type of cell death inducer. In this context, a wide arsenal of stimuli including ER stressors (thapsigargin, tunicamycin, brefeldin), lysosome-targeting agents (bafilomycin A1), mitochondrion-targeting compounds (arsenite, betulinic acid, ceramide), proteasome inhibitors (MG132, lactacystin, ALLN), and DNA damaging molecules (Hoechst 33 342, camptothecin, etoposide, mitomycin) is per se not immunogenic.[5] Conversely, some cytotoxic chemicals that are currently used for anticancer therapy such as anthracyclines, oxaliplatin (but not cisplatin), and cyclophosphamide induce a type of cell death that is immunogenic, yet is accompanied by all known biochemical and morphologic hallmarks of apoptosis.[53,54] Tumor cells that have been killed in vitro with such chemotherapeutic agents elicit a vaccine-like effect when they are injected subcutaneously into immunocompetent mice. This leads to the long-term protection of mice against subsequent rechallenges with live tumor cells of the same type. Cancer cells respond to DNA damaging agents (which constitute an important class of clinically used chemotherapeutics) with a complex signaling pathway that either allows for DNA repair (if the damage is limited) or engages apoptotic mechanisms (if the damage is excessive).[55] Prominent players of the DNA damage response include the ataxia telangiectasia mutated (ATM) and the ATM-related kinases, checkpoint kinases 1 and 2 (CHK1 and CHK2),[56] and the tumor suppressor protein TP53.[57] Beyond their role in the DNA damage response, ATM and CHK1 are known to induce the expression of natural killer (NK) cell group 2D (NKG2D) ligands, thus sensitizing tumor cells to NK-mediated lysis.[58–61] In addition, TP53 might mediate NKG2D ligand-independent immunogenic effects by inducing cell senescence, a status that has been surmised to be recognized by NK cells and macrophages, leading to tumor eradication.[62] Recently, multiple chemotherapeutic agents have been shown to up-regulate the expression of mannose-6-phosphate receptors on the surface of tumor cells, thereby promoting a perforin-independent increase in the permeability to granzyme B released by CD8+ lymphocytes.[63] Altogether, these observations suggest that multiple anticancer agents that are currently used in the clinical setting induce or at least facilitate ICD.

Some chemotherapeutic agents improve anticancer immunity by exerting direct immunomodulatory effects. For instance, cyclophosphamide—which is widely used against lymphomas and leukemia—selectively reduces the frequency of tumor-induced regulatory T cells,[64] induces the differentiation of

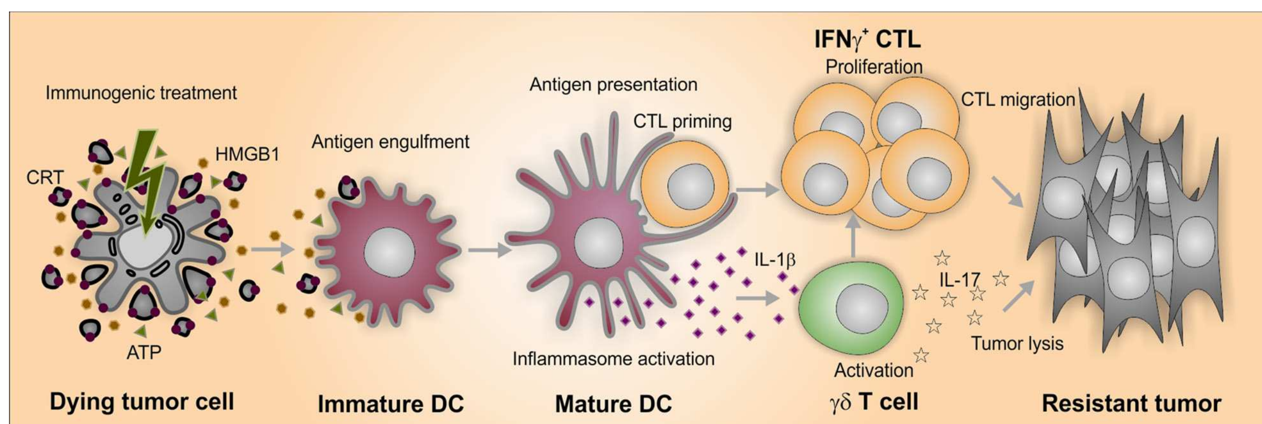

FIGURE 2. Precise orchestration of antitumor T-cell responses elicited following ICD: After (immunogenic) chemotherapy, tumor material is phagocytosed by DCs. These later are also activated by ICD signals emitted by dying tumor cells. Within 2 days, IL-17–producing γδ T cells are recruited to the tumor bed in an IL-1β–dependent manner. Their arrival precedes and correlates with the IFN-γ–producing CD8⁺ T cells infiltration, which is critical for tumor eradication.

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T_H17 cells,[65] enhances the long-term survival and proliferation of lymphocytes,[66,67] and resets DC homeostasis.[54,68–71] Along similar lines, imatinib—a tyrosine kinase inhibitor used in bcr/abl and Kit-induced malignancies—activates NK-dependent antitumor effects in mouse models[72] and stimulates the NK-mediated secretion of IFN-γ in patients with gastrointestinal stromal tumor, thus improving long-term survival.[73] Thus, several chemotherapeutic agents provide therapeutic benefits not only via tumor cell–intrinsic, on-target effects but also by modulating immune responses in an off-target fashion.

RADIOTHERAPY AS A POTENT ANTICANCER VACCINE INDUCER

Focused ionizing radiations induce cancer cell death upon the induction of DNA damage and the overgeneration of reactive oxygen species.[74] For many years, the direct cytotoxic effect of radiotherapy has been considered as the sole determinant of its therapeutic success. However, multiple lines of evidence have accumulated suggesting that the therapeutic effects of radiotherapy cannot be accounted for by tumor cell death alone and hence might depend—at least in part—not only on endothelial cells but also on the host immune system.[75,76] Thus, radiotherapy appears to be more efficient in immunocompetent mice than in their immunodeficient counterparts.[77] Moreover, the irradiation of primary tumors is known to inhibit the growth of nonirradiated metastases that are localized at distant sites (a phenomenon known as "abscopal effect").[75] In line with this notion, the irradiation of primary 4T1 tumors (mouse breast cancer that spontaneously metastasizes) induced a CD8+ T cell–mediated immune response that controlled the growth of lung micrometastases when combined with an inhibitor of cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) receptors (to overcome tumor-induced T-cell tolerance).[78] Recently, these findings have been corroborated in other murine models of cancer, namely, in TSA-derived breast cancer and MCA-38–derived colon carcinoma. In these settings, fractioned radiotherapy (but not single dose) combined to a CTLA-4–blocking antibody led to potent abscopal effects that were paralleled by the production of consistent levels of IFN-γ.[79] Importantly, the frequency of CD8+ T cells elicited by radiotherapy appears to correlate with the intensity of the abscopal effect. Interestingly, radiotherapy influences the chemokine pattern of the tumor microenvironment (promoting CXCL16 secretion by irradiated tumor cells), facilitating the entry of effector CD8+CXCR6+ T cells into irradiated tumor beds.[80] Altogether, these studies demonstrate that radiotherapy induces a T cell–dependent antitumor effect, by inducing and/or recruiting tumor-specific T cells into the tumor bed.[81,82]

Recently, we have demonstrated that tumor cells succumbing upon irradiation elicit a cognate tumor-specific response when injected subcutaneously into syngenic mice, thereby exerting a vaccine-like effect and protecting mice against subsequent challenges with living tumor cells of the same type.[6,7] Irradiation-induced ICD, which might account for, at least part of, the abscopal effect,[83,84] also turned out to rely on the preapoptotic exposure of CRT and the TLR4/Myd88 pathway (see above). Alternatively, it has been suggested that dormant antitumor immunity might get reactivated by radiotherapy-induced inflammation and cytokine release, which together would trigger the recruitment of T cells into the tumor bed.[80,85] Irrespective of the fact that the molecular mechanisms underlying the abscopal effect remain poorly understood, radiotherapy appears as a potent trigger of ICD.

GENETIC BACKGROUND AND CLINICAL OUTCOME

During ICD, TLR4 and P2RX7 on DCs are critical for sensing and decoding the immunogenic message conveyed by the release of HMGB1 and ATP, respectively.[10,12,24] Both *TLR4* and *P2RX7* present several nonsynonymous single nucleotide polymorphisms,[86,87] and loss-of-function TLR4 and P2RX7 mutants (Asp299Gly and Gly496Ala, respectively) display low ligand-binding affinity. In line with the fact that TLR4 plays a critical role during ICD (see above), the *TLR4* Asp299Gly allele has been shown to negatively affect the progression-free survival of breast cancer patients who received anthracycline-based adjuvant chemotherapy[24] as well as of patients bearing colorectal cancers that were treated with oxaliplatin-based regimens.[88] Furthermore, among breast cancer patients who carried wild-type *TLR4*, the *P2RX7* Gly496Ala allele was associated with shortened progression-free survival.[10] These results strongly suggest that the defect in the molecular mechanisms by which ICD is perceived by the immune system limits the efficacy of anticancer chemotherapy. This also provides further support to the concept that anticancer immune responses—and hence all the

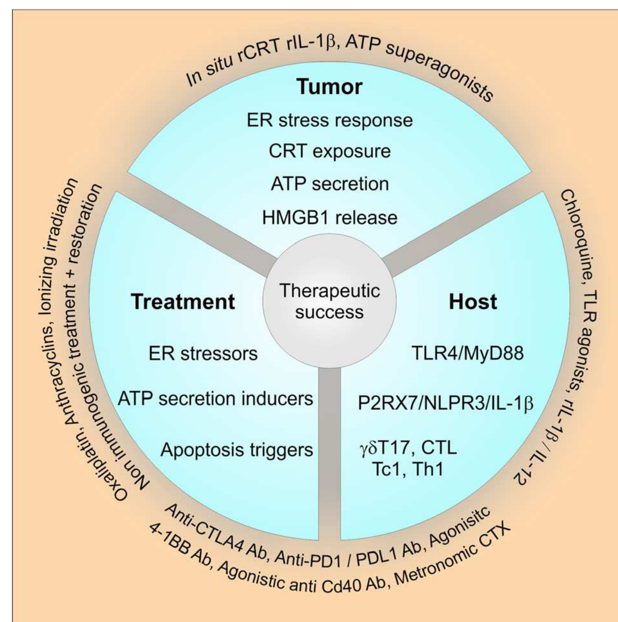

FIGURE 3. Schematic view of tailored anticancer regimens: To achieve therapeutic success, (i) the anticancer regimen should be able to induce ICD, that is, to induce an ER stress before cell death and ATP release. Nonimmunogenic cytotoxic drug can be combined with ER stressors to restore immunogenicity. (ii) The tumor of the patient should have conserved the intrinsic capacities to emit all the immunogenic signals. If not, the defective signals could be identified and then compensate by recombinant CRT or rIL-1β or ATP superagonists. (iii) The loss-of-function mutation of key receptors involved in the perception of ICD signals might also be compensated by triggering alternative TLR pathway or supplementation with the appropriate cytokine. (iv) The combination of immunotherapy and immunogenic chemotherapy enhances the vaccine-like effect of chemotherapy and radiotherapy by overcoming the tumor-induced immunosuppression. Thus, tailored anticancer regimens should be designed by taking into account the genetic background of both the tumor and the host, with an aim to correctly unlock the immune system to obtain the complete eradication of the tumor and long-term tumor-free survival.

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genetic and environmental factors that affect such responses—are crucial for therapeutic success.

In view of these considerations, it is tempting to speculate that detailed information on the patient and tumor genetic background would allow for the design of tailored anticancer regimens with optimal efficacy and limited adverse effects. In particular, such information might (at least partially) predict the proficiency of a tumor to undergo ICD and elicit a cognate immune response and, if required, suggest the development of interventions aimed at restoring the immunogenicity of cell death. For instance, defects in the ER stress module that is required for CRT exposure during CRT might be corrected by the direct absorption of recombinant CRT to the tumor.[6] Along similar lines, *TLR4* loss-of-function mutations (which result in the deficient perception of HMGB1-conveyed signals) might be compensated by combining chemotherapy with alternative TLR agonists or with lysosomal inhibitors such as chloroquine,[24] whereas defects in P2RX7 signaling might be reverted by the administration of exogenous IL-1β[10] or apyrase inhibitors.

Alternative approaches for the elicitation of an anticancer immune response focus on the reversal of tumor-induced tolerance by means of immunomodulatory agents such as monoclonal antibodies targeting suppressive pathways (such as CTLA-4, PD-1, Lag3, Tim-3) or engaging activating receptors (such as CD40, CD27, 4-1BB), cytokines, and cell based-approaches (T and DC) in combination with conventional therapies.[89–91] In preclinical models, these strategies have been shown to enhance the vaccine-like effect of both chemotherapeutic and radiotherapeutic regimens, thereby constituting promising approaches.[91;79] Gulley and colleagues[92] reported that, in prostate cancer patients, the combination of radiotherapy with an admixture of a recombinant vaccine against prostate-specific antigen (PSA) and B7.1 costimulatory molecules induced a significant increase in PSA-specific and MUC1-specific T cells. The expansion of MUC1-specific T cells indicates that a tumor antigen cross-priming occurs in vivo following radiotherapy. Brody and colleagues reported a phase I/II trial where low-dose radiotherapy combined with in situ TLR9 agonists induced lymphoma remission, not only at the site of the treated lesion but also at distant sites, associated with detectable anticancer T-cell responses.[93]

Taken together, these studies suggest that efficient anticancer regimens should combine immunogenic chemotherapy or radiotherapy with immunomodulatory agents that overcome tumor-induced immunosuppression. Moreover, whenever required, the defects in the molecular machinery for the execution and perception of ICD should be compensated to obtain the complete eradication of tumors and long-term tumor-free survival (Fig. 3).

PERSPECTIVES

As we have discussed above, some (but not all) chemotherapeutic and radiotherapeutic regimens induce the immunogenic death of tumor cells that, in specific circumstances, lead to the elicitation of a potent anticancer immune response. This vaccine-like effect is critical for both therapeutic success and long-term tumor-free survival. The ability of anticancer drugs to induce an ER-stress response that precedes cell death, the intrinsic capacity of tumor cells to emit immunogenic CDAMPs in a defined spatiotemporal order, and the ability of the host to perceive these signals and to overcome tumor-induced immunosuppression appear as fundamental prerequisites for the vaccine-like effect of radiotherapy or chemotherapy. We believe that future anticancer regimens should be tailored to each patient and tumor's genetic background to take into account all these elements, as this will result in combination therapies with optimal cytotoxic and immunogenic profiles and limited adverse effects.

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3. DISCUSSION

There is compelling evidence that successful chemotherapy accounts for both tumor-related factors and host immune responses. The immune contribution is elicited in two ways by conventional therapies.

On one hand, some therapeutic programs can elicit specific cellular responses — beyond the stereotypical apoptotic pathway — that render tumor-cell death immunogenic. These immunogenic modifications include: pre-apoptotic calreticulin translocation, induction of expression of MHC molecules, tumor specific antigens or death receptors, and post-apoptotic HMGB1 secretion. Other chemotherapeutic agents that induce immunogenic tumor-cell death may elicit yet more mechanisms. On the other hand, some drugs may have side effects (beyond their effect on the tumor itself) that stimulate the immune system, through transient lymphodepletion, the subversion of immunosuppressive mechanisms and the direct or indirect stimulatory effects of immune effectors.

Here, they have been shown the local and systemic effects of CTX leading to the induction of antitumor immunity *in vivo*, and the synergism with type I IFN. For the first time, in my knowledge, it has demonstrated that beyond its effect on DC homeostasis, CTX can and does induce a wide-spread tumor apoptosis with strong immunogenic features. The immunogenicity of CTX-induced cell death is proven 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 MAFOSFAMIDE (MAFO). 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 ecto-CRT 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. Fourth, when injected into immunocompetent mice, MAFO-EG7 cells worked as a therapeutic vaccine, thus protecting 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 [1].

Although DC loaded with MAFO-EG7 cells could stimulate CD8 T-cell cross-priming, the addition of type I IFN greatly enhanced this process. In agreement with the *in vitro* results, type I IFN administered *in vivo* strongly synergized with CTX for tumor eradication. Because type I IFN treatments were done in the local tumor microenvironment, I foresee that the beneficial effect of these cytokines may reflect an action at the DC–tumor interface.

Cross-presentation of cell-associated Ag, such as dead cell-derived Ag, is a crucial process for generating CD8 T cell responses to Ag that are not expressed by APC, such as viruses that do not infect APC or tumors of non-hematopoietic origin [2]. Among APC, DC are specialized for cross presentation and accumulating literature indicates that *in vivo* this process is mainly fulfilled by the CD8α^+ DC subset [3]. In the steady state, CD8α^+ DC constitutively cross present self Ag, such as material derived from apoptotic cells as a result of constitutive cell turnover, leading to self-tolerance [4]. However, in the context of infection or pathological distress, signals consisting of microbial compounds or of inflammatory stimuli released by cells of innate immunity act as danger signals that shape DC functions, thus leading to their activation/differentiation, in a process referred to as “licensing” of DC to cross-priming [5]. The findings I’ve recently reported demonstrate that type I IFN can act as a powerful promoter of CD8 T cells cross-priming against apoptotic cell-derived Ag both by “licensing” DC and by enhancing cross-presentation, thus switching the tolerogenic response by CD8α^+ DC into an immunogenic one. In doing so, type I IFN control CD8α^+ DC activity at three distinct levels. First, type I IFN treatment prolongs the intracellular persistence of antigenic particles engulfed by phagocytic CD8α^+ DC, resulting in enhanced Ag cross-presentation, as evidenced by surface expression of MHC-I/OVA-peptide complexes and by induced OT-I cell proliferation.

Antigen persistence is a crucial event regulating the magnitude of cross-presentation and is promoted by a reduced lysosomal proteolysis that delays the degradation of phagocytosed Ag, in a process requiring a limited phagosomal acidification. As a mechanism regulating intraphagosomal pH, the NOX2 enzyme was shown to induce active alkalinization of the phago-lysosomal compartments selectively in DC. As showed, Ag persistence induced by type I IFN strongly correlated with pH alkalinization and was restrained by addition of the NOX2 inhibitor DPI, resulting in reduced cross-presentation of EG7-derived OVA by CD8α^+ DC. Previous studies reported that signaling through TLR2, TLR3, TLR4 and TLR9 enhance Ag uptake resulting in more efficient cross-presentation [6]. It has been shown that type I IFN enhance CD8α^+ DC cross-presentation of tumor apoptotic cell-derived Ag mainly affecting Ag processing. Consistent with this view, studies on human DC indicate that type I IFN can affect the expression of a

number of genes associated with processing as well as the expression of inducible proteasome subunits [7, 8]. It is worth mentioning that in our setting, withdrawal of apoptotic EG7 (apoEG7) tumor cells from the co-culture at 3h did not prevent IFN-induced Ag retention and OVA cross-presentation in CD8α$^+$ DC, provided that type I IFN were maintained in the culture for the remaining 15h. In fact, removal of both apoEG7 cells and type I IFN after the 3h culture resulted in only partial Ag retention and no DC activation and cross-presented OVA. This observation suggests that type I IFN exposure may be required all through the Ag processing phase to lead to MHC-I cross-presentation and DC activation. As a second effect, type I IFN promoted the survival of phagocytic, but not of non-phagocytic, CD8α$^+$ DC, through the selective modulation of the apoptosis-related genes Bcl-2 and Bcl-xL. This finding suggests an elegant regulatory mechanism by which type I IFN selectively sustain the life span of Ag-bearing DC for induction of effective immune responses, while favoring a rapid clearance of steady-state DC [9]. DC lifespan critically regulates the efficiency of cross-priming and the outcome of adaptive immunity, although little is known about the role of Ag persistence in this process [10]. A recent study in mice infected with bacillus Calmette-Guérin has shown that Ag persistence in infected DC is strictly correlated with DC survival [11].

Altogether, these data suggest that duration of Ag persistence and survival of DC may be two linked processes regulating the extent of Ag presentation and cross-presentation. Thus, the effects of type I IFN in promoting both Ag persistence and survival of CD8α$^+$ DC may be two tightly correlated events, although which one is causative of the other remains to be determined. A third process accounting for type I IFN effects is the activation of DC, revealed by upregulation of both co-stimulatory markers and pro-inflammatory cytokines, that provide a license signal for DC to cross-priming, consistent with previous reports [12, 13]. Multiple events have been described licensing DC for cross-priming that include CD40L engagement by CD4 T helper cells, stimulation by NK cells, TLR triggering and exposure to soluble factors released upon injury or infection [14]. Among these soluble mediators, type I IFN have been described to be particularly efficient in inducing cross-priming in a CD4 T cell-independent manner implying a faster immune reaction [13, 15]. Besides the appreciated effects in promoting cross-priming against soluble or viral Ag, some recent evidence suggests that type I IFN may also affect cross-presentation of cell-associated Ag [7]. Of note, a recent study on the newly-described mouse merocytic DC subset has shown that these cells are endowed with potent ability to prime both CD4 and CD8 T cells against tumor cell-associated Ag partly through their ability to produce type I IFN upon engulfment of apoptotic tumor cells [16]. Moreover, cellular association of dsRNA with irradiated EG7 cells was shown to elicit CD8 T cell responses *in vivo* that were dependent on dsRNA-induced type I IFN secretion by DC [17].

In agreement with the *in vitro* results shown in this thesis, it has been recently shown that intratumoral administration of IFNα strongly synergizes with systemic immunotherapy for induction of anti-tumor response, involving enhanced DC cross-presentation [18]. Another recent study from Schreiber and colleagues claimed an obligate role for endogenous type I IFN in naturally occurring, host-protective immune responses against many highly immunogenic tumors [19, 20]. More recently, the same group has demonstrated that type I IFN exert their activity early during the development of the antitumor response, and that their major physiological function is directed selectively toward a single host cell population (i.e., DC), and that, at least in part, they function to enhance the capacity of CD8α$^+$ DC to cross-present antigen to CD8 T cells [21].

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 [22-24]. My results show that CTX, at non-myeloablative doses, despite inducing transient reduction of total BM cells [22, 25], spares DC precursors (DCP), which, instead, increase in their relative frequency (day 3 post injection), 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 [26, 27]. 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 [23]. Remarkably, CTX-mediated DCP mobilization critically required endogenous type I IFN, induced soon after CTX treatment systemically [28, 29] and in the local BM environment. Recent reports showed that type I IFN reactivate dormant hematopoietic stem cells, promoting their proliferation and mobilization *in vivo* [30, 31]. In addition, type I IFN can directly stimulate the turnover of DC *in vivo*, especially of CD8α$^+$ DC, and promote the generation of DC from BM precursors [9, 12].

The findings here reported support the role of type I IFN in homeostasis, with crucial implications for patients undergoing myelodepleting regimens, as concomitant treatment with IFNα could accelerate recovery of immune competence [32]. Importantly, although type I IFN 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 type I IFN to succeed [33-35]. In this regard, because type I IFN was recently shown to reduce cell 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 ablation [36].

Another interesting finding reported herein is the enhanced tumor infiltration by DC following CTX treatment. Although it cannot be 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 [37-39]. 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-OVA-peptide complexes. Of note, the presence of $CD11c^+$ DC co-expressing MHC-I-OVA-peptide 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 [40-42]. 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 [40, 41, 43].

After the peak of tumor infiltration, considerable numbers of DC migrated to tumor draining lymph nodes (dLN) in CTX-treated mice (day 10 post injection) 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 [44]. It has been proposed that migratory DC, rather than $CD8\alpha^+$ DC, retain more immunogenic features, thus enhancing immune responses in naïve CTX-treated mice [24]. 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 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 $CD8\alpha^+$ DC, previously expanded by CTX, to initiate antitumor responses. In this scenario, co-administration of type I IFN in the local intratumoral milieu functions as a powerful signal that licenses DC for efficient cross-priming.

Currently, CTX represents the gold standard immunomodulatory chemotherapeutic drug, and the antitumor efficacy of its combination with immunotherapy has long been studied in preclinical models[33, 45-47], as well as in clinical trials [48-51].

Altogether, the reported 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. Furthermore, the reported study provides new knowledge on the effect of type I IFN in promoting intracellular Ag persistence in CD8α+ DC that have engulfed apoptotic tumor cells, and in favoring their survival (*vs* non-Ag bearing DC), with the final result of enhancing cross-presentation of apoptotic tumor cell-derived Ag by CD8α+ DC. The above mentioned, newly described, effects strongly support the observed powerful capability of CTX and type I IFN to synergize in the induction of antitumor responses *in vivo*.

This study provide new knowledge on type IFN I and CTX, old molecules whose newly described properties can be exploited for the design of innovative clinical protocols in which the generation of effective antitumor immunity is crucially required.

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