The Biology Behind

Immunogenic Cell Death and Cross-Priming Are Reaching the Clinical Immunotherapy Arena

Commentary on Saji et al., p. 2568

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Photodynamic Therapy Synergizes with Intratumoral Injection of Dendritic Cells

In this issue of Clinical Cancer Research, Saji et al. (1) show the preclinical therapeutic efficacy of combining photodynamic therapy and intratumoral injection of dendritic cells (DC) in s.c. transplanted tumors. The investigators have used systemic doses of a hydrophilic photosensitizer and a laser beam directed at s.c. malignant lesions implanted in mice. Once this procedure had been done, bone marrow–derived DC were injected inside the experimental tumor lesions. Tumors derived from two different cell lines have been treated with this regime, and compelling data has shown synergistic effects of the therapeutic combination for both locally treated tumor and distantly implanted lesions. Additional experiments showed that the therapeutic effects correlate with a cellular immune response against tumor antigens that includes detection of cytolytic T lymphocytes that, if adoptively transferred, protect naïve recipient mice from tumor challenge. The cellular and molecular mechanisms involved in the induction of these highly efficacious therapeutic effects have not been addressed, but are likely related to the biological phenomena of tumor antigen cross-priming and immunogenic cell death. These mechanisms are now an important focus of research in immunology, among other reasons because of their promising implications to attain synergistic combinations of radiotherapy and/or chemotherapy with immunotherapy. The most exciting points raised by this article from the group of Edgar Engleman are the potency of the treatment on the poorly immunogenic B16 melanoma model and the feasibility of the approach for clinical translation.

Cross-Presentation of Tumor Antigens

The term cross-priming denotes that the induction of cellular immunity against cell-associated antigens requires the presentation of those antigens by professional antigen-presenting cells. This concept was inferred from experiments showing that, when immunizing with different cell sources, antigens were presented by variants of the MHC-presenting molecules expressed by bone marrow–derived cells of the host, which were genetically absent in the immunizing cell (2). Extensive experiments in mice indicate that the professional players in the game of cross-presentation belong to DC populations (3).

Antigens can be presented to specific T cells by different pathways. MHC class II molecules specialize in presenting antigens that have entered the cells in endosomes as a consequence of phagocytosis or pinocytosis. Peptide products digested by lysosomal enzymes are presented on the cell surface to CD4+ T cells by professional antigen-presenting cells. Endogenously translated cytosolic peptides generated by the proteasome machinery are transported to the endoplasmic reticulum to be associated with nascent MHC class I molecules that, when reaching the plasma membrane, present antigen to cognate CD8+ T cells.

Classically, the class II pathway, which is restricted to DC, B cells, and macrophages, process and present exogenously captured antigens, whereas the class I pathway would present peptides that are primarily translated in the cell that presents the antigen. There exist DC, however, that are capable of redirecting cell-exogenous, internalized antigens to the class I antigen-presenting pathway via incompletely understood subcellular translocation mechanisms. These functions permit the access of endosome proteins to the cytosol where they are digested by the proteasome, thereby entering the MHC class I loading pathway. Researchers in this field also use the term cross-priming to define the process that leads to immunization against MHC class I–restricted antigenic determinants when the peptide comes from an extracellular protein, regardless of whether this protein is soluble or associated with a third party cell. It is important to consider that DC differentiated in culture from monocytes or bone marrow precursors in the presence of growth factors are capable of mediating this function, but little is known about which subpopulations of DC in vivo are endowed with this capability of cross-priming antigen to CD8+ T cells (4). A mouse subpopulation of lymph node–resident CD11c+ DC that expresses CD8α has been shown to be critical for cross-priming soluble antigens. The human equivalent subpopulation of DC has not been defined.

To meet T cells, cross-priming DC need to migrate to lymphoid organs via lymphatic vessels, a highly regulated process that involves chemokines being produced both in the lymphoid organs and in the lymphatic vessel’s endothelium. Once in the T cell area of the lymphoid tissue, each DC has the chance to interact with thousands of T cells scanning for those that recognize the antigens presented on the DC surface.
Increasing evidence indicates, however, that incoming DC need the contribution of DC populations that reside in the lymph node to actually present antigen (5). It has been experimentally proposed that there exists a DC network in lymphoid tissue that should permit the exchange of antigens and antigen-presenting molecules (6).

As a result of these processes, antigens expressed in peripheral cells such as malignant cells, can be presented (cross-presented) in lymphoid tissue by DC to different subsets of T cells (7) via MHC class I, class II, and possibly CD1 pathways. The result of presentation by default under baseline conditions is the induction of abortive T cell activation, which leads to tolerance (cross-tolerance). Under inflammatory, septic, or viremic conditions, however, cross-presentation would result in T cell clonal expansion and acquisition of effector functions, including those of tumor-specific cytolytic T lymphocytes.

Intratumoral injection of DC is a strategy that expects the intratumorally released DC to take up antigens, process them through the MHC class I and II pathways, and carry them to local draining lymph nodes (8).

The source of tumor cell antigens for cross-presentation is an issue for debate. There is experimental evidence for the role of heat shock protein chaperones (gp96 and HSP70) bound to cell-derived peptides that are internalized by DC through efficient receptor-mediated internalization (9). These heat shock proteins could be released from dead cells in the tumor once the plasma membrane is disrupted. Other investigators pinpoint whole proteins as the source of cross-presented peptides (10).

In both cases, apoptotic bodies could indeed be an extraordinary self-service buffet of potential tumor antigens to feed artificially injected DC inside the tumor, when a factor promoting or inducing malignant cell death is given prior to or concomitantly with a local injection of DC. Obviously, DC should be protected from the tumor-killing agent if it were not selective for tumor cells. Injecting DC once the potential deleterious activity of this factor is cleared is the most reasonable approach.

In humans, compelling evidence for cross-presentation of tumor antigens has been achieved by analyzing T cell specificities against mesothelin-derived peptides in patients with pancreatic cancer who had been repeatedly immunized with allogeneic tumor cells transfected to produce granulocyte colony-stimulating factor. Informative cases showed the presentation of peptides by MHC class I molecules that were absent in the allogeneic vaccine cells, but were expressed in host cells of the patient (11).

### DC Maturation/Activation Determines Cross-Priming

As mentioned before, the default outcome of cross-presentation is the induction of tolerance (cross-tolerance) by means of deletion or anergy of the relevant T cells (12). Antigen-presenting DC need to be activated to become immunogenic. A set of signaling pathways defines a gene expression program known as DC maturation or activation. Maturation is triggered by pathogen-associated patterns detected by innate receptors on DC, proinflammatory cytokines, and CD40 ligation engagement with cognate, activated CD40-ligand+ helper T cells. Most changes in signaling and in the transcriptome of DC during activation are shared by different stimuli, but there are features that are selectively turned on by each type of maturation factor. Such differences are likely aimed at fine-tuning the most appropriate type of immune response.

DC maturation/activation results in (a) acquisition of higher levels of expression of surface costimulatory molecules such as CD80 and CD86; (b) secretion of cytokines such as tumor necrosis factor, interleukins (IL)-15, IL-1, IL-6, IL-12, IFNs and proinflammatory chemokines; (c) fostering DC migration to lymphoid tissue; (d) down-regulation of phagocytosis, processing and cross-presentation of new antigens, accompanied by augmented expression of MHC antigen-presenting proteins intensely directed to the plasma membrane.

The immune mechanisms that destroy tumor cells are similar to the cytolytic functions that control viral infection. The recognition of viral infection by DC relies on the detection of features of viral nucleic acids. Internalized viral DNA is recognized in the endosomes by toll-like receptor (TLR)-9, whereas RNA with viral characteristics are recognized in the endosomes by TLR-3, TLR-7, and TLR-8. These pathways of detection of viral molecules in endocytosed material seem to be very important in cross-presentation/cross-priming. In addition, viral RNA, and possibly DNA, can be recognized by cytosolic nucleic acid binding proteins such as Pkr, Rigi, and Mda-5. This intracellular pathway is cleverly deployed to detect direct viral infection of the DC (13). Both routes of viral detection induce the production of type I IFNs (mainly IFNα and IFNβ). Indeed, type I IFNs have been found to be of great importance for cross-priming, as well as for a potent cytolytic T lymphocyte induction (14). In addition, virally induced maturation triggers the production of IL-12, a key mediator for the orchestration of cellular immune responses that include cytolytic T lymphocytes, T helper 1, and natural killer cells. Therefore, enforcing the immunogenic features of acute viral infection in tumor tissue is thought to be useful for cancer immunotherapy.

In the study by Saji et al. (1), the choice of immature versus mature DC is probably wise because fully mature DC are not capable of cross-presenting internalized proteins (15). In their study, however, this remains only as a testable hypothesis because the investigators have not yet compared mature DC versus immature DC.

### Not All Cell Deaths Are Equal: What is the Meaning of Immunogenic Cell Death?

What is the factor triggering DC maturation in aseptic conditions? It is widely admitted that apoptosis is seen as harmless by the immune system and leads to immunologic ignorance or tolerance (16). Two morphologic types of cell death have been defined. Apoptotic death generally involves mitochondrial dysfunction and nuclear fragmentation, whereas the plasma membrane is still preserved. By contrast, necrotic death is characterized by plasma membrane disruption as an early event. In the apoptotic process, a cascade of intracellular cysteine proteases (caspases) is sequentially activated, and gives rise to the events that terminate the cell in a self-inflicted manner. Cellular remains known as apoptotic bodies are rapidly internalized by macrophages in what is known to be an immunologically silent process, but they can also be internalized by DC giving rise to specific immunity or tolerance (2, 7, 16).
At the present time, however, there is much excitement about the idea that cell death induced by certain chemotherapeutic agents and radiotherapy may be immunogenic, in spite of morphologically resembling apoptosis (17). This predicts the existence of endogenous danger signals that would activate the maturation program in DC that have uptaken cell debris. Inducible alarm signals could include any substance made or modified by distressed or injured cells, but absent from cells dying in physiologically normal processes (18). The chemical nature of the danger signals produced by cells dying under stressful conditions is not well understood, but could involve, for example, heat shock proteins or features of the degraded cellular nucleic acids that could resemble the viral counterparts (19).

In a recent elegant study, Casares et al. (20) showed that the use of an anthracycline (i.e., doxorubicine) provokes an immunogenic cell death not directly mediated by the drug, but rather by caspase-dependent features of the cell debris. These investigators have found that γ irradiation causes, to some extent, this type of alarm-sounding death, and that doxorubicine-killed tumor cells are internalized more avidly by DC.

All in all, there is tremendous enthusiasm in combining conventional therapies with immunotherapy. This is somewhat counterintuitive because antimitotics ought to impair the clonal expansion of lymphocytes, a necessary function of adaptive immune responses. Apart from inducing immunogenic cell death, however, chemotherapy can enhance cancer immunity by a number of mechanisms being actively explored at the present time, including (a) decreases in the numbers of regulatory/suppressor T cells, (b) debulking the number or cancer cells for a subsequent immune attack, (c) modifying tumor tissue in such a way that it becomes more accessible to inflammatory infiltrates, (d) curtailing malignant cell production of immunosuppressive factors. Recently published clinical

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Fig. 1. Apoptotic or necrotic cancer cell death achieved by conventional cancer therapies enhances antigen transfer to DC. In the presence of either microbial stimuli (pathogen associated patterns, PAMP) or in the presence ill-defined endogenous danger signals, DC that have uptaken potential tumor antigens become activated or mature. Reaching lymphoid tissue, these mature or activated DCs migrate and cross-present tumor antigens to T cells, resulting in cross-priming of an effector antitumor immune response. There is increasing evidence that lymph node–resident DCs are involved in cross-priming, implying antigen transfer between incoming and resident DCs. TLR, toll-like receptors; HSP, heat shock proteins.

1 N. Casares, personal communication.
trials suggest that this concept can improve the therapeutic effects of chemotherapy combinations (21).

Which kind of cell death is induced by photodynamic therapy is an issue that has not been addressed in the models studied by Saji et al., but it is tempting to speculate that it would locally provide an immunogenic environment (7, 17).

**Intratumoral Injection of DCs**

If the tumor environment can be rendered a good source of immunogenic antigens for cross-presentation, then local numbers of DC could be a limiting factor. The simplest strategy to achieve an infiltrate of DC inside malignant lesions is repeated direct injection of DC suspensions. The alternative strategies are gene therapy approaches that achieve local production, such as granulocyte macrophage colony-stimulating factor and macrophage inflammatory protein-3α (8).

Once DC are artificially released in a malignant lesion, they are expected to endocytose antigens from dead or even living cells, become mature, migrate to draining lymph nodes and present antigen to specific T lymphocytes (Fig. 1). Other studies have found that intratumoral injection of DC in mice pretreated with chemotherapy (22) or radiotherapy (23) increases the therapeutic effectiveness. Even clinical trials consisting of intratumoral injection of DC have been done (24), including injection of IL-12-transfected DC, showing the feasibility, safety, and biological effects of procedures which still have a very limited rate of clinical responses (25).

Intratumoral injection of DC is clinically feasible because good manufacturing practice procedures of DC differentiation in culture have been developed with excellent safety records. Intratumoral injection of DC by itself, however, is not very efficacious in rodent tumor models. Two general approaches have been envisioned to increase antitumor immunity: (a) induction of tumor cell death with chemotherapy or radiotherapy, and (b) transfecting DC with genes that improve their performance at initiating and sustaining the antitumor immune response (8). In Saji et al.’s work, the effects of photodynamic therapy are crucial to favor local and systemic immunity after intratumoral DC injection. There are no morphologic or functional observations of the fate of DC after injection inside the tumor, or regarding their interplay with cancer tissue or its dead remains. Previous experiments consisting of intratumoral injections of DC engineered to express IL-12 showed that DC with internalized apoptotic bodies could be traced to local lymph nodes (26). Tumor cells and the malignant stroma produce factors that decrease the performance of intratumoral DC interfering with maturation/activation and migration. Among them, the most relevant are transforming growth factor-β, IL-10, vascular endothelial growth factors, prostaglandin E2, and IL-8. The effectiveness of the approach of intratumoral injection of DC would benefit from diminishing the influence of such factors. A simple way to achieve this might be with the local destruction of tissue which is expected to occur in photodynamic therapy.

Overall, this first report on photodynamic therapy plus intratumoral DC injection shows promising efficacy. Nonetheless, the mechanistic insight must be strengthened to make the most of it in further development. In particular, future investigations should address the type of cell death, the mechanisms of antigen transfer to intratumoral DC, and the efficiency of DC migration. Deeper and comparative analysis of the elicited immune responses and the tumor-rejection leukocyte infiltrates will also be of much interest. The potential for autoimmunity as a result of unwanted cross-presentation of a key self antigen is always an issue when taking into account these types of immunotherapeutic interventions. The investigators have observed vitiligo in mice being treated for B16 melanoma. This is very interesting because other strategies that are based on the intratumoral injection of DC have not yet reported symptomatic self-reactivity.

Regardless of the incomplete mechanistic insight, the study by Saji et al. offers a practical alternative for the treatment of cutaneous tumors and, conceivably, for metastatic uveal melanoma, because photodynamic therapy is common clinical practice in ophthalmology. Intraoperative application of the technique for visceral tumors is also possible for clinical translation when surgical resection is not possible. Impressive preclinical efficacy and clinical feasibility should encourage further development of this combined strategy.

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**References**

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