

EXPERT
REVIEWS

Adjuvant role for cell death during chemo- and radiotherapy of cancer?

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Evaluation of: Apetoh L, Ghiringhelli F, Tesniere A *et al.* Toll-like receptor 4-dependent contribution of the immune system to anti-cancer chemotherapy and radiotherapy. *Nat. Med.* 13, 1050–1059 (2007).

Chemotherapy and radiotherapy are supposed to mediate their anticancer activity via the direct elimination of tumor cells. Dying cells, however, also release molecules that promote the activation and the functional maturation of the most potent antigen-presenting cells, the dendritic cells. Mature dendritic cells are endowed with the ability to cross-prime T cells against the antigens contained in the dead cells. This paper evaluates the results from a recent article in which the success of some protocols for anticancer therapy depends, besides their direct cytotoxic effect, on the induction of a specific immune response: the effect is mediated both in mice and humans through the release by dying tumor cells of the nuclear protein high-mobility group 1 protein and by its interaction with Toll-like receptor 4. Larger studies on various therapeutic regimens are needed to evaluate the general relevance of this observation.

KEYWORDS: antigen presentation • cell death • HMGB1 • immunosurveillance • response to chemotherapy and radiotherapy • Toll-like receptors • tumor immunology

The transfer of antigens to professional antigen-presenting cells (APCs) and the presence of signals that trigger their maturation are the two key events for the induction of an efficient T-cell response. Identical requirements restrict the development of an efficient immune response against tumors. A recent study suggests that anticancer chemotherapy and radiotherapy generate both tumor-associated antigens and signals sufficient to activate APCs and to trigger innate and adaptive antitumor immune responses [1].

The transfer of intracellular antigens among living cells is supposed to be a rare event. By contrast, cell death associates with the release in the microenvironment of intracellular antigens in a particulate form, which represents a preferential substrate for internalization by dendritic cells (DCs) [2,3]. Internalized dead cell-derived antigens are processed in dedicated intracellular vesicles and cross-presented to T cells. This paradigm has been defined for cells committed to death upon viral infection [4]. Other evidence, however, including that in the paper under evaluation [1], indicate that tumor cell death after

conventional chemo- and radiotherapy allows the transfer of antigens expressed in neoplastic tissues to DCs.

For a long time, apoptosis has been considered a silent modality of cell death, which does not elicit an innate or adaptive immune response, and is possibly instrumental for the induction of peripheral tolerance [5]. However, 'pure' apoptosis is probably uncommon *in vivo*; by contrast, postapoptotic secondary necrosis, that is the modality of cell death that characterizes uncleared apoptotic cells, has apparently a dual effect. On the one hand, it triggers local inflammation that in turn favors neoangiogenesis and wound healing. Thus, in the context of solid tumors, inflammation may promote growth and progression [6]. On the other hand, the release of intracellular pro-inflammatory signals contributes to the onset of adaptive immune responses against antigens contained inside the dead cells [2,7]. Tumor cell death for example is capable of promoting an antitumor immune response; this apparently depends on the stimulus used to activate the death pathway. Thus, tumor cell death

induced by anthracyclines or x-rays promotes a cytotoxic T-lymphocyte response that confers permanent antitumor immunity [8].

Various molecules have been identified that, when released in the environment, amplify and sustain the function of APCs and the establishment of T-cell-dependent immune responses [9]. These molecules are referred to as 'alarmins' or damage-associated molecular patterns (DAMPs): they comprise an array of structurally diverse host molecules that are rapidly released during infection or tissue damage and include defensins, eosinophil-derived neurotoxin, cathelicidins, heat shock proteins and the high-mobility group 1 protein (HMGB1) [10].

HMGB1 is a 30-kD nonhistone nuclear protein, expressed in all eukaryotic cells and highly conserved through evolution (99% identity in mammals). HMGB1 enacts important functions

within the nucleus and most cells contain high amounts of this protein. Nuclear HMGB1 binds without sequence specificity to the minor groove of DNA and enables physical interactions between DNA and various factors, including p53, NF- κ B and steroid hormone receptors. In addition to the nuclear location, some cells express HMGB1, also referred to as amphoterin, on the plasma membrane. Membrane-associated HMGB1 controls neurite outgrowth smooth muscle cell chemotaxis and tumor cell metastasis [10].

HMGB1 reaches the extracellular environment either passively after cell necrosis [11] or after active secretion by cells of the innate immune system (FIGURE 1) [12]. HMGB1 released from necrotic cells triggers: the production of proinflammatory cytokines, including TNF- α , from monocytes [11,13]; and activates the NF- κ B pathway, an event critical for entry of vessel-associated stem cells into damaged/necrotic tissues and

for tissue regeneration [14]. These results indicate that HMGB1 contributes to the inflammation elicited by cell and tissue necrosis. Extracellular HMGB1 also triggers the functional maturation of professional APCs and their ability to present soluble and corpuscolate antigens [7,15]. Indeed, HMGB1 released from necrotic cells behaves as an immune adjuvant for soluble and particulate antigens [7].

The receptor for advanced glycated endproducts (RAGE) was the first receptor for HMGB1 to be identified. RAGE is a member of the immunoglobulin superfamily expressed by monocytes and macrophages, DCs, endothelial cells and vascular smooth muscle cells [16]. Subsequently, Toll-like receptor (TLR)2 and TLR4 have also been proposed to bind HMGB1. The ability of HMGB1 to interact with multiple innate receptor, possibly dependent to its structural conformation, is possibly important for its 'alarming' function [17–19].

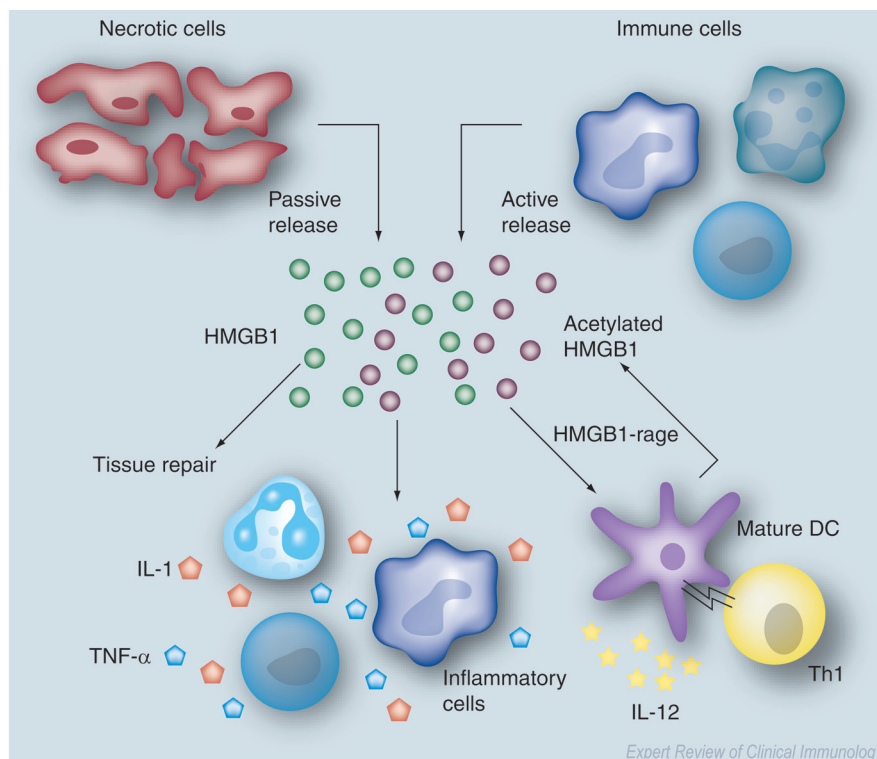


Figure 1. Release of HMGB1 in the extracellular space. In most cells, HMGB1 is a mobile nuclear protein that constantly shuttles between the nucleus and the cytosol. There are two main conditions in which HMGB1 reaches the extracellular environment: passive release from necrotic cells or active secretion from cells of the innate immune system. Necrotic cells have leaky plasma membranes and HMGB1 diffuses into the surrounding milieu. Active secretion of HMGB1 from immune cells, such as monocytes, macrophages, DC and NK cells, occurs following activation by microbial and proinflammatory stimuli. This leads to acetylation of specific lysine residues, which blocks the import of HMGB1 into the nucleus. Cytosolic HMGB1 is then packaged in secretory vesicles before being released into the environment. Extracellular HMGB1 is important for tissue repair, for the recruitment of inflammatory cells and for the DC cross-priming of Th1 cells. These effects are mediated by binding to membrane receptors, such as RAGE and possibly TLRs, and vary with the target cells. DC: Dendritic cell; HMGB1: High-mobility group 1 protein; NK: Natural killer; RAGE: Receptor for advanced glycated endproducts; TLR: Toll-like receptor.

Methods

The study addresses four main issues:

- Do radiotherapy and chemotherapy trigger tumor-specific immune responses?
- What is the role of TLR4 expressed by APCs?
- Is the phenomenon dependent on the release of alarmins by dying tumors and which is, in particular, the role of HMGB1?

Table 1. Role of the immune system in chemo radiotherapy induced tumor rejection.

Tumor models	Treatment	Dose and schedule	Antitumor efficacy*	
			WT	Nu/Nu
GOS	ip. oxaliplatin	5 mg/kg single injection	++	–
GOS	ip. irinotecan	120 mg/kg two injections (over 24 h)	++	++
TS/A	X-ray irradiation	10 G single irradiation	+++	+/-
P03	ip. docetaxel	60 mg/kg single injection	+++	+++
CT26	it. doxorubicin	100 µg single injection	+++	+/-
MCA205	X-ray irradiation	10 G single irradiation	+++	+++

* ++++, ++, +/- and – indicate good, moderate, poor and no efficacy of anti-tumor treatment, respectively.

CT26: Colon adenocarcinoma; GOS: Glasgow osteosarcoma; ip.: Intraperitoneally; it.: Intratumorally; MCA205: Fibrosarcoma; Nu/Nu: Nude athymic mice; P03: Pancreatic adenocarcinoma; TS/A: Mammary adenocarcinoma; WT: Wild-type.

- Do *TLR4* polymorphisms play a role in the long-term response of cancer patients to chemotherapy and radiotherapy?

To address the first question, the authors compared the effect of antineoplastic agents on various cancers in immuno-competent or immunodeficient *nu/nu* mice (models in which T cells were required for the effect of some agents are summarized in TABLE 1).

To investigate the role of TLRs, the authors compared the efficacy of chemotherapy and radiotherapy in wild-type versus TLR-deficient mice (*Tlr1-7*, *9*^{-/-} mice). TLRs transduce signals through two pathways involving two distinct adapters, Toll/IL-1R domain containing adapter-inducing IFN (TRIF) and myeloid differentiation primary response protein (MyD88) [20]. To evaluate the relevance of such adapters in the antitumor effect of conventional therapies, the authors evaluated tumor growth and the response to therapy in both *Trif*^{-/-} and *Myd88*^{-/-} mice.

To evaluate the role of secreted HMGB1, the authors used siRNAs or neutralizing anti-HMGB1 antibodies.

To investigate the role of *TLR4* polymorphisms, the authors assessed, by coimmunoprecipitation, the binding of HMGB1 to the normal *TLR4* allele and to a *TLR4* loss-of-function allele. The ability of DCs bearing the normal or the mutant *TLR4* alleles to cross-present tumor antigens to T cells was also compared. Finally, the impact of the *TLR4* allele expression on the response to anthracyclines of breast cancer patients was evaluated.

Results

The authors report that tumor cells dying as a consequence of chemotherapy or radiotherapy treatments elicit cognate immune responses that contribute to tumor regression *in vivo* (FIGURE 2). T-cell priming by dying tumor cells required *TLR4* expression DCs both *in vitro* and *in vivo*.

HMGB1 was released by dying tumor cells and bound to *TLR4*. The interaction between HMGB1 and *TLR4* was not required for the phagocytosis of dying tumor cells by DCs or for

DC activation; however, *TLR4* engagement by HMGB1 controls the processing and presentation of tumor cell-associated antigens; in fact *Tlr4*^{-/-} DCs degraded the antigenic material so swiftly that they failed to present derived epitopes to T cells.

Finally, mutations of *TLR4* that affect its binding to HMGB1 influenced the immunological outcome of conventional (anthracycline-based) chemotherapy in human cancer. In particular, DCs derived from individuals bearing the variant *TLR4* allele cross-presented poorly the MART-1 tumor antigen from dying melanoma cells to MART-1-specific T cells. The altered cross-presentation ability conferred by the *TLR4* Asp299Gly was restored by treatment with chloroquine, a drug that prevents the lysosomal degradation of internalized antigens, and that also overcomes the defect of antigen presentation by *Tlr4*^{-/-} mouse DCs *in vitro* and *in vivo*.

A cohort of 280 female breast cancer patients presenting with lymph node involvement and treated with surgery, irradiation and anthracycline-based chemotherapy were genotyped for *TLR4*. Patients who exhibited a variant *TLR4* allele did not differ from patients displaying the normal *TLR4* allele for all classical prognostic factors (age, pathological tumor size, lymph node involvement, tumor grade, hormone receptors and median follow-up). The metastasis-free survival was significantly longer in the cohort carrying the normal allele of *TLR4*.

Discussion & significance

Chemotherapy and radiotherapy act not only by the direct destruction of tumor cells but also because they trigger an immune response that is required for the success of therapy. This immune response mediates the suppression of tumor growth and determines the long-term survival of animals and possibly of patients. In this study, the authors define a molecular mechanism (albeit possibly not the only one) that makes tumor cell death immunogenic, namely the interaction between HMGB1 released from dying tumor cells and *TLR4* expressed by antigen-presenting DCs.

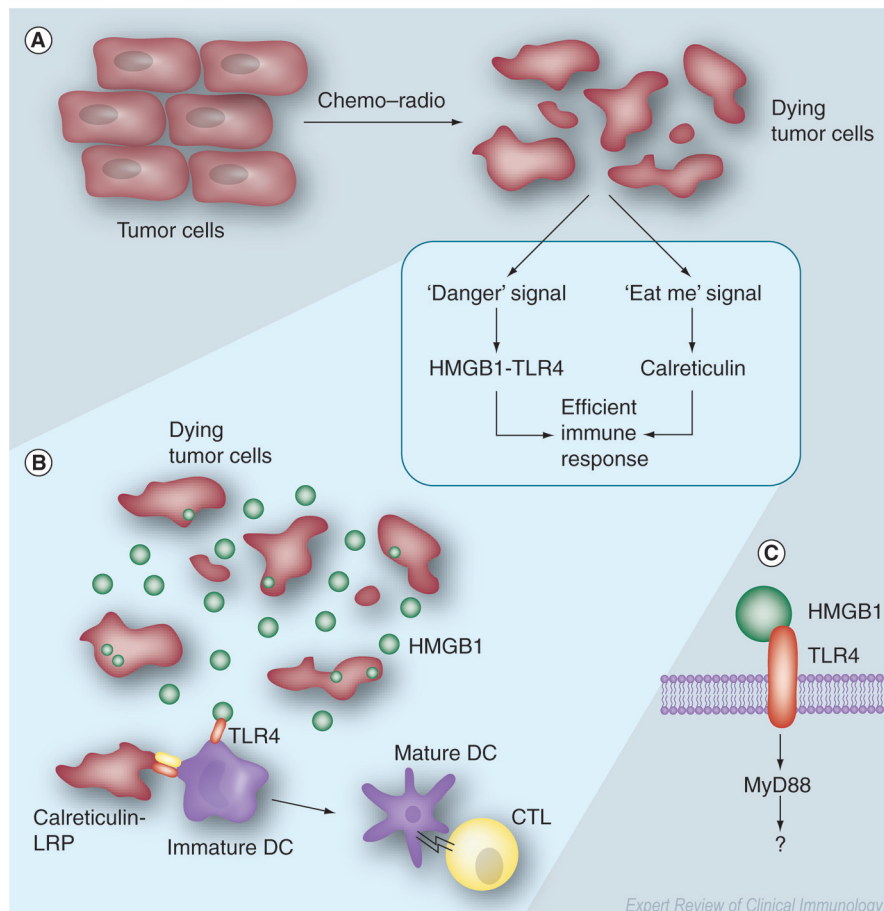


Figure 2. Tumor cells dying in consequence of radio- and chemotherapy can elicit an efficient immune response that depends on HMGB1 and TLR4. (A) A phagocytic tag is generated by the dying tumor cell (e.g., calreticulin exposure on the surface of dying cell). Calreticulin acts by binding and activating the LRP on the DC. HMGB1 released by dying tumor cells acts as an alarmin. (B) HMGB1 binding to TLR4 allows the successful cross-priming of the antigen-specific T cells. (C) HMGB1-TLR4 binding exerts its effects through MyD88.

CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; HMGB1: High-mobility group 1 protein; LRP: Low density lipoprotein receptor-related protein; MyD88: myeloid differentiation primary response protein 88; TLR: Toll-like receptor.

The results indicate that TLR4 activation delays the degradation of the phagocytic cargo within DCs, thereby allowing for optimal antigen presentation. The exact mechanism by which HMGB1-TLR4 interactions influence the processing and presentation of tumor antigens is not completely clarified. Finally, the authors show that the *TLR4* polymorphism Asp299Gly (and the cosegregating missense mutation Thr399Ile; data not shown), which is found in 8–10% of Caucasians, compromises the efficacy of anticancer chemotherapy, at least in breast cancer. The *TLR4* Asp299Gly single-nucleotide polymorphism reduces the interaction between TLR4 and HMGB1 and abolishes the capacity of DCs to activate, *via* cross-presentation, Mart1-specific T cells. This is the first report revealing that immunogenetic factors might influence clinical outcome in breast cancer patients treated with chemotherapy and radiotherapy.

Altogether, the authors propose a model in which the immunological effect of radiotherapy and chemotherapy must satisfy two major requirements: the exposure of a phagocytic tag (calreticulin) [21] and the release of an alarm signal (HMGB1) by dying tumor cells. These two codes are required to license DCs, interacting with dying tumor cells, for antigen uptake and TLR4-dependent antigen processing, respectively. Only when both codes are correctly emitted by dying tumor cells and perceived by DCs, can an immune response ensue. This knowledge may be clinically exploited to enhance the immunogenicity of current chemotherapeutic regimens, possibly including combining chemotherapy with the administration of different TLR agonists or with lysosomal inhibitors.

Expert commentary & five-year view

The recent publication by Apetoh and colleagues [1] represents the first observation that an immunological response plays a role in the outcome of conventional radiotherapy and chemotherapy for cancer. This does not apply to all treatments, but occurs specifically when some stimuli are used to activate death pathways in cancer cells (TABLE 1). To induce an immunological response is not an end point when clinicians design a treatment. This possibility needs to be extensively verified in various clinical settings, since it could represent an actual breakthrough for more effective antineoplastic therapies.

The authors suggest that a hierarchy of events underlies the immunogenicity of dying cells:

- Synchronized tumor cell death as a consequence of the treatment
- Effective release of HMGB1
- DC expression of *TLR4* alleles able to activate the Myd88 intracellular pathway after interaction with HMGB1

All steps could represent future targets for intervention aimed to enhance the immunological consequences of radiotherapy and chemotherapy for cancer. In particular, drugs capable of inducing/amplifying the release of HMGB1 and TLR agonists directly delivered at the site of tumor cell death could represent valuable additions to conventional therapeutic schemes.

The identification of *TLR4* alleles that restrict or amplify the immunogenicity of dying cells could facilitate the selection of patients to be enrolled in or excluded by neoadjuvant

radiotherapy and chemotherapy. These approaches are often only effective in a fraction of patients: a molecular approach that allows the identification of potential responders would be valuable in the clinics.

It must also be mentioned that a similar code (phagocytic tag + alarm signal) could be implicated in the pathogenesis of systemic autoimmune diseases, including in particular, systemic lupus erythematosus, in which the immunogenic death of normal cells is critical [22,23].

Some questions are still open and need to be clarified in the future, in particular, whether acetylated HMGB1, actively secreted by DCs or activated macrophages, mediates the same effects revealed for the nonacetylated molecule released by dead tumor cells? It is also important to understand if the coexistence on the same macromolecular structure of both tumor antigens and HMGB1 is a prerequisite for the induction of an anticancer immune response. Authors do not extensively characterize the type of tumor cell death induced by the treatment. Apoptosis is likely to occur, possibly followed by secondary necrosis [24]. HMGB1 released as a consequence of secondary necrosis can be complexed to chromatin; the role of these complexes in the cross-presentation of tumor antigens needs to be further characterized.

Financial & competing interests disclosure

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Key issues

- Conventional therapies for cancer induce an efficient antitumor immune response.
- The nuclear protein high-mobility group 1 protein (HMGB1) is a key signal that triggers the antitumor immune response.
- Toll-like receptor (TLR)4 expression by dendritic cells is instrumental for the development of the antitumor immune response.
- The interaction between HMGB1 released by dying cells and TLR4 expressed by dendritic cells is a requirement for the presentation of tumor-associated antigens to T cells.
- *TLR4* polymorphisms influence the efficacy of anticancer chemotherapy.

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- of considerable interest

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