Molecular Interactions between Dying Tumor Cells and the Innate Immune System Determine the Efficacy of Conventional Anticancer Therapies

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Abstract
The efficacy of anticancer treatments is mostly assessed by their ability to directly inhibit the proliferation of tumor cells. Recently, we showed that tumor cell death triggered by chemotherapy or radiotherapy initiates an immunoadjuvant pathway that contributes to the success of cytotoxic treatments. The interaction of high mobility group box 1 protein (HMGB1) released from dying tumor cells with Toll-like receptor 4 (TLR4) on dendritic cells was required for the crosspresentation of tumor antigens and the promotion of tumor-specific cytotoxic T-cell responses. Breast cancer patients harboring the loss-of-function Asp299Gly polymorphism of TLR4 relapsed earlier after receiving anthracycline-based chemotherapy. These data suggest that HMGB1- and TLR4-dependent immune responses elicited by conventional cancer treatment may increase the probability to achieve a durable therapeutic success.

Immune-Mediated Effects of Anticancer Treatments
Most anticancer treatments target tumor cells and rapidly proliferating cells of the immune system without any distinction. The resulting immunosuppression has discouraged oncologists from performing extensive studies on the role of the immune system in anticancer therapies. However, evidence from mouse experiments indicates that some anticancer agents such as doxorubicin could favor the activation of immune effectors cells (1). Casares and colleagues (2) reported that anthracyclines induced the immunogenicity of cancer cells in mice, a process that was dependent on the induction of caspase-dependent tumor cell death leading to antigen uptake by dendritic cells (DC) and to the activation of CD8+ cytotoxic T cells. More recently, Obeid and colleagues (3) showed that plasma membrane exposure of calreticulin accounts for the immunogenicity of anthracyclines and irradiation in vivo.

Nevertheless, the preclinical screening for new anticancer drugs still relies on human xenografts transplanted into immunodeficient mice. Although this approach is indeed relevant to study the cell-autonomous effects of anticancer agents, it completely overlooks any contribution of the host immune system to tumor eradication (4). To examine the implication of T cells in the success of chemotherapy or radiotherapy protocols, we compared the ability of a panel of anticancer treatments to promote tumor regression in immunodeficient versus competent mice. This strategy allowed us to identify therapeutic protocols in which tumor insult promotes adaptive immune responses (5).

High Mobility Group Box 1 Protein Binding on Toll-Like Receptor 4 Is Required for Crosspresentation of Tumor Antigens
Tumor cell death triggered by cytotoxic agents may lead to the release of danger signals that favor the development of anticancer immunity (5, 6). Although Toll-like receptors (TLR) were originally described to detect microbial products (7), it has recently been shown that endogenous danger signals from dying cells can also bind TLRs and, thus, modulate adaptive immune responses (8). Therefore, we hypothesized that tumor cell death triggered by radiotherapy or chemotherapy could induce TLR-dependent T-cell immunity. In a large series of experiments, we comparatively monitored the action of doxorubicin, irradiation, and oxaliplatin on established colon carcinoma (CT26), mammary adenocarcinoma (TS/A), or Glasgow osteosarcoma (GOS) tumors, respectively, in wild-type and tlr4−/− mice. Although cytotoxic treatments significantly inhibited tumor growth in wild-type hosts, they had no or significantly less pronounced effects on tumor development in tlr4−/− mice, underlining the absolute requirement of TLR4 in the control of tumor growth (9). T cells were also mandatory for the observed antitumor effects because nude mice failed to mount an efficient antitumor response after local radiotherapy or systemic chemotherapy of TS/A or GOS tumors, respectively (9).

After ligand binding, TLR4 conveys intracellular signaling mainly via two adaptor proteins, Toll/IL-1R domain containing adaptor inducing IFNβ (TRIF; ref. 7) and myeloid differentiation primary response protein (MyD88; ref. 7). We therefore tested which among these adaptors would be involved in the antitumor efficacy of oxaliplatin against GOS. Oxaliplatin was efficient against GOS tumors established in wild-type or Trif−/− mice, yet failed to act against tumors growing in MyD88−/− hosts (9). Altogether, these data suggest that the TLR4/MyD88 pathway contributes to the success of anticancer therapy.

To unravel the molecular mechanisms underlying the immunogenicity of anticancer treatments in vivo, we screened the TLRs implicated in the immunogenicity of dying tumor cells. Dying EG7 cells (that are EL4 thymoma cells expressing OVA protein) injected
into wild-type or tlr1<sup>-/-</sup>, 2<sup>-/-</sup>, 3<sup>-/-</sup>, 5<sup>-/-</sup>, 6<sup>-/-</sup>, 7<sup>-/-</sup>, and 9<sup>-/-</sup>-deficient mice could elicit the priming of OVA-specific naïve T cells in vivo. However, no T-cell priming was observed when dying cells were injected into tlr4<sup>-/-</sup> mice, indicating that the immunogenicity of anthracyclines, X-ray, or oxaliplatin-treated tumor cells was dependent on TLR4 (9, 10). In line with these results, tlr4<sup>-/-</sup>-deficient bone marrow–derived DCs were selectively impaired in their ability to present tumor antigens from dying tumor cells to T cells in conditions in which wild-type DC were able to do so. We subsequently verified in vivo that TLR4 dictated the crosspresentation of membrane-associated tumor antigens (but not soluble antigens) to T cells (Fig. 2; ref. 9).

Because the absence of TLR4 negatively affected antigen presentation, we examined putative alterations within the antigen-processing machinery of tlr4<sup>-/-</sup> DCs compared with wild-type DCs. We found that, within tlr4<sup>-/-</sup> DCs, phagocytosed EG7 particles were more rapidly destroyed via the lysosomal pathway than in wild-type DCs, a finding that is in agreement with a previous study reporting that TLR4 prevents the fusion between endosomes and lysosomes (11). To strengthen this hypothesis, we performed additional in vitro antigen presentation experiments on tlr4<sup>-/-</sup>-deficient DCs, and we were able to restore antigen presentation by adding either chloroquine (a lysosomotropic alkaline) or bafilomycin A1 (a specific inhibitor of the vacuolar ATPase responsible for lysosomal acidification). Finally, the combined treatment of oxaliplatin and chloroquine was equally efficient in controlling EL4 tumor growth in wild-type and tlr4<sup>-/-</sup> mice, meaning that chloroquine administration could compensate the defective response of tlr4<sup>-/-</sup> hosts to chemotherapy (9).

Numerous endogenous danger signals ferried by dying tumor cells to innate immune effectors may account for the immunogenicity of tumor cell death (5, 8, 12). To identify the danger molecule(s) that contribute to the immunogenicity of dying tumor cells, we systematically compared the release of putative TLR4 ligands by live tumor cells, oxaliplatin-treated EG7 cells, or doxorubicin-treated CT26 cells. We found that high mobility group box 1 (HMGB1) molecules but not other known TLR4 ligands (including heat shock proteins, fibrinogen, fibronectin, and β-defensin 2) were selectively present in the supernatant of dying CT26 or EG7 tumors. These data suggested that the nuclear molecule HMGB1, which has been described to act as an alarmin (13), may determine the immunogenicity of dying tumor cells. The binding of HMGB1 to TLR4 was previously shown using fluorescence resonance energy transfer analyses and immunoprecipitation studies (14). To delineate the in vivo relevance of the interaction between TLR4 and HMGB1, we transfected CT26, EG7, and MCA205 fibrosarcoma cells with a siRNA designed to knockdown HMGB1. This manipulation revealed that the release of HMGB1 was mandatory for the priming of T cells by dying tumor cells. HMGB1 knockout also hampered the capacity of
doxorubicin-treated CT26 tumor cells to confer protection against rechallenge with untreated tumor cells. Furthermore, a neutralizing antibody directed against HMGB1 blunted antigen presentation of tumor antigens from dying EG7 cells and inhibited T-cell priming elicited by dying EG7 cells in vivo (9). We concluded that the interaction of HMGB1 released by dying tumor cellswith the TLR4 receptor present on DCs dictated the outcome of anticancer therapy. However, as addition of recombinant HMGB1 alone could not mimic the DC maturation induced by apoptotic tumor cells, we are currently searching for additional signals delivered by tumor cells to DCs that could contribute to efficient T-cell differentiation and Th1 polarization.

Clinical Relevance of TLR4 in the Response to Anthracyclines

A human tlr4 single nucleotide polymorphism (SNP) rs4986790 has been associated with decreased responses to the prototypical TLR4 ligand lipopolysaccharide (15). The single nucleotide substitution (+896A/G) in the tlr4 gene leads to the replacement of an aspartic acid by a glycine (Asp299Gly) in the extracellular domain of TLR4. The pioneering study of Arbour and colleagues (15) has shown that alveolar epithelial cells from individuals bearing the Asp299Gly variant form of the receptor lost their capacity to produce IL-1α after lipopolysaccharide stimulation. By transfecting human HeLa cells (which express the normal form of TLR4) with a cDNA encoding the Asp299Gly allele of TLR4, we could show that the expression of the variant form of TLR4 significantly decreases the binding of recombinant HMGB1 to TLR4, as determined by coimmunoprecipitation (9). We further verified that the expression of TLR4 Asp299Gly in HeLa cells leads to impaired nuclear factor-κB activation after stimulation with recombinant HMGB1. Moreover, although monocyte-derived DCs (MD-DC) derived from normal individuals could crosspresent antigens from dying melanoma cells to CTL in an HMGB1-dependent manner, MD-DC from individuals bearing the TLR4 Asp299Gly mutation failed to do so (9).

To investigate the clinical relevance of the TLR4 mutation for the response to anticancer treatments, we designed a retrospective cohort of 280 breast cancer patients presenting with lymph node involvement and treated with local radiotherapy and anthracycline-based adjuvant chemotherapy. We first genotyped the patients of this cohort by performing Taqman PCR analyses on blood DNA. The frequency of monoallelic expression of the Asp299Gly polymorphisms in this cohort was 17.1%. No significant difference for all classic prognostic factors (age, pathologic tumor
size, lymph node involvement, tumor grade, hormone receptors, and median follow up) was noted between normal individuals and patients bearing the mutated \textit{tlr4} allele. However, metastasis-free survival was significantly decreased in women carrying the variant allele of \textit{tlr4} (50% of relapse in mutated versus 37.4% in nonmutated patients at 10 years; Log-rank test, \(P = 0.03\); ref. 9). Altogether, this study identified the \textit{tlr4} mutation as an independent predictive factor for the success of anthracycline-based adjuvant regimen (Fig. 3).

**Prospects for the Clinical Use of Anticancer Compounds**

Drug developers and clinical oncologists tend to give most credit to anticancer drugs that mediate the strongest antiproliferative effects against tumor cells, based on the assumption that these drugs mainly act via cell-autonomous effects. In this study, we propose that the immune system of the host makes a decisive contribution to the efficacy of anticancer therapies. We have shown in four tumor models constrained by three independent therapeutic regimens that an intact immune system was required for optimal antitumor effects. The action of these anticancer agents on tumors promoted the development of innate and adaptive immune responses. HMGB1 was identified as one of the danger signals released from agonizing tumor cells which, via its binding on TLR4, was responsible for an efficient crosspresentation of tumor antigens. Finally, we showed that individuals with the Asp299Gly polymorphism of \textit{tlr4} exhibit a higher risk of relapse after treatment by anthracyclines-based chemotherapy and local radiotherapy.

The data presented in this study question the current clinical management of cancer patients. For instance, high-dose glucocorticoids are commonly prescribed to treat some side effects of anticancer treatments such as vomiting. However, glucocorticoids are potent immunosuppressors (4). Thus, administering such drugs to patients may annihilate the putative immunoadjuvant effect induced by anticancer agents. Similarly, it might be worth favoring neoadjuvant over adjuvant chemotherapy, as the former may lead to an increased delivery of tumor antigens resulting in an enhanced immune response against cancer. Finally, the functional relevance of the \textit{tlr4} SNP, which affects 10% of Caucasians, deserves further attention. We showed that chloroquine could correct deficient crosspresentation by TLR4-mutated mouse bone marrow DC or human TLR4 Asp299Gly MD-DC and that in \textit{tlr4} \textsuperscript{-/-} mice, chloroquine synergized with oxaliplatin. Furthermore, a clinical study on glioblastoma-bearing patients reported a benefit in adding chloroquine to conventional chemotherapy and radiotherapy (16). This suggests that combining chloroquine with conventional anticancer treatments may increase the chances of success of anticancer regimens, in particular in individuals bearing the \textit{tlr4} mutation. An alternate method to compensate for TLR4 deficiencies may rely on the administration of other TLR ligands to boost

![Figure 3](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-08-1027-sup-03-01)

**Figure 3.** The clinical consequences of the TLR4 polymorphism are illustrated. HMGB1 has a reduced affinity for TLR4 in patients bearing the Asp299Gly allele. Supplementation of chemotherapies with chloroquine might restore antigen presentation by DC and, hence, stimulate a potent cytotoxic T-cell response.
antitumor immune responses. Thus, we found that administration of specific ligands of TLR3 or TLR9 after irradiation could restore potent antitumor effects in \textit{tlr4}-deficient mice (10). It is our hope that the clinical implementation of these therapeutic strategies will improve the efficacy of existing therapeutic regimens (17).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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