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The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy

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Summary: For the last four decades, the treatment of cancer has relied on four treatment modalities, namely surgery, radiotherapy, cytotoxic chemotherapy, and hormonotherapy. Most of these therapies are believed to directly attack and eradicate tumor cells. The emerging concept that cancer is not just a disease of a tissue or an organ but also a host disease relies on evidence of tumor-induced immunosuppression and polymorphisms in genes involved in host protection against tumors. This theory is now gaining new impetus, based on our recent data showing that optimal therapeutic effects require the immunoadjuvant effect of tumor cell death induced by cytotoxic anticancer agents. Here, we show that the release of the high mobility group box 1 protein (HMGB1) by dying tumor cells is mandatory to license host dendritic cells (DCs) to process and present tumor antigens. HMGB1 interacts with Toll-like receptor 4 (TLR4) on DCs, which are selectively involved in the cross-priming of anti-tumor T lymphocytes *in vivo*. A TLR4 polymorphism that affects the binding of HMGB1 to TLR4 predicts early relapse after anthracycline-based chemotherapy in breast cancer patients. This knowledge may be clinically exploited to predict the immunogenicity and hence the efficacy of chemotherapeutic regimens.

Keywords: *TLR4, HMGB1, dendritic cell, anti-tumor vaccination, chemotherapy*

Introduction

Both for the treatment of disseminated cancer and for the cure of minimal residual disease (micrometastases), chemotherapy is the main treatment, despite the severe side effects and the minimal expectations of curing metastases. The oncological armamentarium has been bolstered in the last 5 years by the introduction of molecularly targeted therapies and anti-angiogenic agents (1). The clinical development of immunotherapy strategies is also encountering a renaissance with the emerging Toll-like receptor (TLR) agonists (2), the anti-cytotoxic T-lymphocyte antigen-4 (3), and programmed death-1 (4) antibodies and refined vaccines and adoptive therapies. The time has come to propose combination therapies, based on the knowledge of the immunostimulatory side effects of chemotherapeutic agents and the immunoadjuvant effects of tumor cell death.

We have known for decades that for a vaccine to elicit an efficient immune response, adjuvants are required, i.e. molecules that stimulate antigen-presenting cells (5, 6). Janeway's (7–9) extended self versus non-self model proposed that immune responses would be triggered by microbes through a set of pattern recognition receptors that bind to conserved 'pathogen-associated molecular patterns' (PAMPs). However, there is evidence, as first reported by Matzinger (10), that antigen-presenting cells would recognize endogenous danger/alarm signals from distressed, injured, or damaged tissues. The danger model suggests that self-molecules can act as 'damage-associated molecular patterns' (DAMPs). Injured or damaged tissue can trigger acute and transient immune responses against self-antigens (11, 12), because dying cells release adjuvant factors that amplify and sustain dendritic cell (DC) and T-cell-dependent immune responses (13–15). Uric acid has been identified as an endogenous immune adjuvant (16). *In vitro*, uric acid stimulated immature DCs to express costimulatory molecules (16). *In vivo*, it augmented cytotoxic T-lymphocyte (CTL) responses to immunizations with particulate antigen but not to peptide-pulsed activated DCs. Extracellular nucleotides are important regulators of inflammation and immune response. Nucleotides released by regulated exocytosis or passive leakage after cell damage bind to P2 purinergic receptors expressed on DCs, thereby promoting interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) secretion and inhibiting T-helper 1 cell differentiation (17). Recently, the roles of interferon (IFN) type 1 and the *N*-ethyl-*N*-nitrosourea-induced germline mutation 3d were described in the T-cell-dependent immunogenicity of dying splenocytes expressing a membrane-associated form of ovalbumin (OVA) (14, 15).

Several DAMPs, including hyaluronans, heat shock proteins (HSPs), and fibronectin, have been described to be ligands of TLRs (18) in the context of autoimmune tissue destruction, atherosclerotic lesions, or during the degradation of extracellular matrix (19). However, endogenous 'danger signals' thus far have not been implicated in anti-tumor immune responses. Here, we show that dying tumor cells promoted by cancer therapies trigger a cognate immune response in a TLR4-dependent fashion and that TLR4 triggering requires HMGB1 released by dying tumor cells.

The immune system participates in tumor regression during chemotherapy or radiotherapy

Until now, no one has addressed the question of whether the immune system might contribute to tumor eradication induced by chemotherapy. The history of drug screening may explain why this question has not been tackled before. Systematic drug screening began in 1955 at the National Cancer Institute (NCI) using rapidly dividing murine hematological malignancies (L1210 and P388 leukemias) (1, 20). In an attempt to find drugs active against solid tumors, the NCI changed strategies and adopted the xenografting of about 60 human tumor cell lines into immunodeficient hosts (21–23). While these screening systems allowed for the linking of the expected mechanism of action with the growth-inhibitory patterns of the anticancer drugs against tumor cell lines, they failed to establish correlates between the latter and clinical outcome in patients. Indeed, the study of human tumor kinetics following xenografting into immunocompromised animals simply overlooks the contribution of T cells [for nu/nu and severe combined immunodeficient (SCID) mice], B cell (for SCID mice), and/or macrophage (for SCID NOD mice)-dependent host defenses against tumors.

We first screened a large panel of mouse tumor cell lines for their *in vivo* sensitivity to anticancer agents (such as doxorubicin, docetaxel, oxaliplatin, and ionizing radiation) in both immunocompetent or nu/nu BALB/c or C57BL/6 mice, in an attempt to evaluate the role of T cells in the anti-tumor effects mediated by such cytotoxic compounds. Some but not all

Table 1. **Immunogenicity of conventional cytotoxic treatments**

				Anti-tumor efficacy*		
Tumor models	Treatment	Administration route	Dose and schedule	WT	Nu/Nu	TLR4$^{-/-}$
GOS	Oxaliplatin	Systemic (i.p.)	5 mg/kg single injection	++	–	–
GOS	Irinotecan	Systemic (i.p.)	120 mg/kg two injections (over 24 h)	++	++	ND
TS/A	X-ray irradiation	Local	10 G single irradiation	+++	+/–	+/–
P03	Docetaxel	Systemic (i.p.)	60 mg/kg single injection	+++	+++	ND
CT26	Doxorubicin	Intratumoral	100 μg single injection	+++	+/–	+/–
MCA205	X-ray irradiation	Local	10 G single irradiation	+++	+++	ND

GOS, Glasgow osteosarcoma; TS/A, mammary adenocarcinoma; P03, pancreatic adenocarcinoma; CT26, colon adenocarcinoma; MCA205, fibrosarcoma; i.p., intraperitoneally; ND, not done; WT, wildtype.
*+++, ++, +/–, and – represent good, moderate, poor, and no efficacy of anti-tumor treatment, respectively.

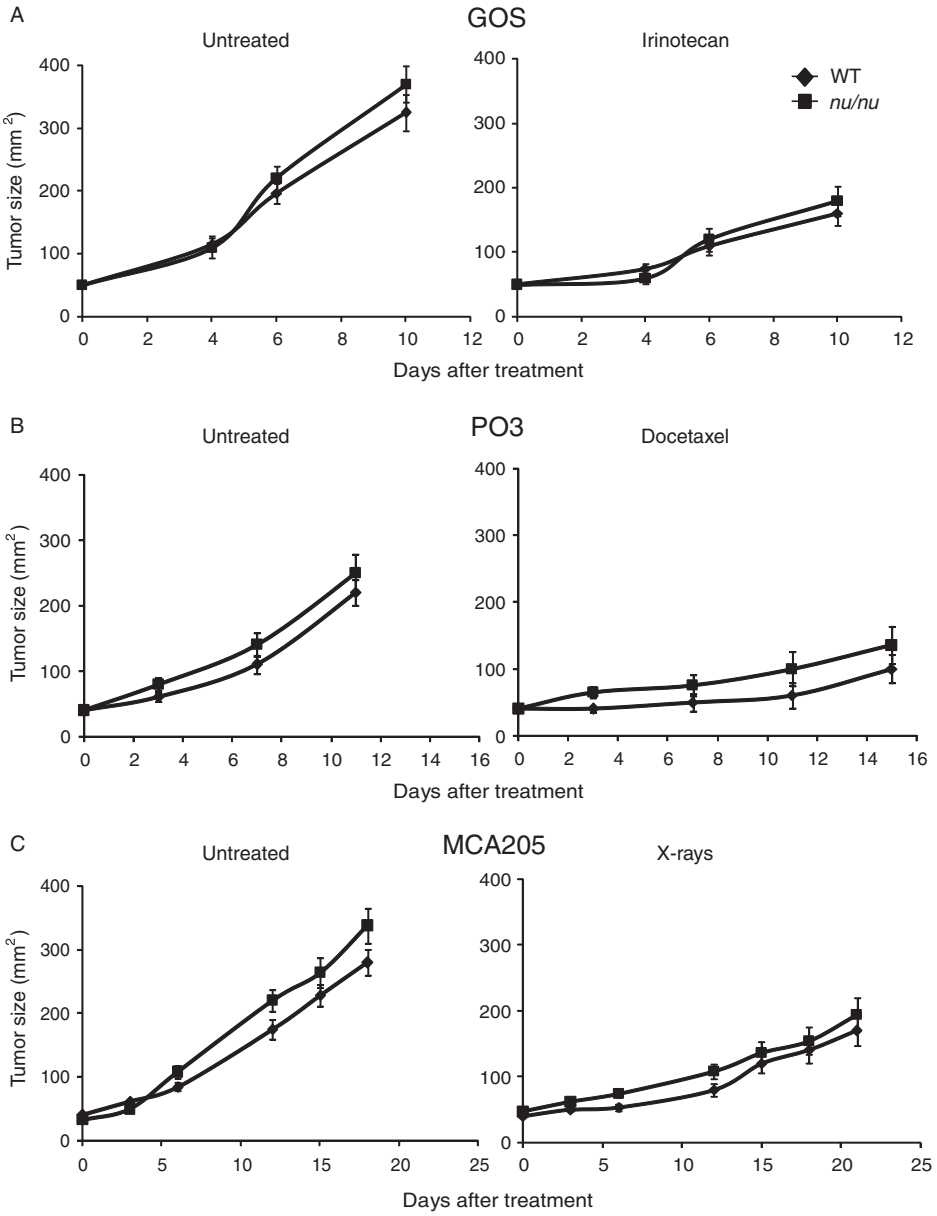

Fig. 1. **T-cell-independent antitumor efficacy of chemotherapy or radiotherapy.** (A, B) Glasgow osteosarcoma and P03 pancreatic adenocarcinoma tumors were heterotransplanted in both immunocompetent and athymic (nu/nu) mice. When the tumors became palpable, mice were either left untreated or were treated by systemic chemotherapy (irinotecan 120 mg/kg i.p. at day 5 and 6 for GOS and docetaxel 60 mg/kg i.p. at day 10 for P03). (C) MCA205 tumors were injected into the right flank of immunocompetent and nu/nu mice. When the tumor reached 40 mm^2, mice were either left untreated or were treated by local X-ray irradiation (10 Grays). Results (mean tumor size ± SEM, five mice per group) are representative of three independent experiments. $^*P < 0.05$.

protocols revealed a critical role for T cells in the observed anti-tumor effects (*Table 1*). For instance, the doxorubicin-induced regression of established CT26 tumors was more pronounced and sustained in immunocompetent mice than in nu/nu littermates (24). TS/A breast cancers reaching 30–50 mm^2 diameter were highly susceptible to 10 Gray-irradiation in immunocompetent but not in nu/nu mice (25). The growth of the heterotransplanted osteosarcoma Glasgow osteosarcoma (GOS) was hampered in immunocompetent mice treated with systemic oxaliplatin but not in nu/nu littermates (25). However, in some other instances, such as treatment of established GOS, pancreatic P03 and MCA205 sarcoma with irinotecan (26), docetaxel (26), or X-rays, respectively, the anti-tumor effects were T-cell independent (*Fig. 1*). Altogether, these data suggested that insult to tumor cells can elicit cognate immune responses that contribute to tumor regression after chemotherapy or radiotherapy.

Contribution of TLR4 to the efficacy of anti-cancer drugs

Because cognate immune responses largely depend on innate immunity (7), we investigated the innate effector mechanisms that could account for T-cell activation during chemo- or radiotherapy. To address whether TLRs could be involved, we compared the efficacy of chemotherapy- or radiotherapy-induced antitumor effects in wildtype (WT) versus TLR-deficient hosts. TLR4 has previously been reported to play a part in lung tumorigenesis caused by chemically induced pulmonary inflammation (27). Because this observation did not link TLR4 expression to the induction of specific anti-tumor immune responses, we started to investigate the efficacy of doxorubicin, X-rays, and oxaliplatin on established CT26, TS/A, and GOS tumors, respectively, comparing the results achieved in WT and *Tlr4*$^{-/-}$ mice. In all three tumor models, TLR4 was consistently required for preventing tumor outgrowth upon systemic chemotherapy or local radiotherapy (25). Upon recognition of their ligands, TLRs transduce signals through two pathways involving two distinct adapters, TRIF (Toll/IL-1R domain containing adapter-inducing IFNβ) and MyD88 (myeloid differentiation primary response protein), which is used by all TLRs except TLR3 (28). Hence, we investigated the relevance of such adapters in the anti-tumor efficacy mediated by oxaliplatin against GOS. Oxaliplatin could promote tumor growth delay in WT as well as *Trif*$^{-/-}$ but not in *Myd88*$^{-/-}$ mice, where no tumor retardation was observed, a result also obtained in *Tlr4*$^{-/-}$ littermates.

To delineate the mechanism underlying the TLR4-dependent anti-tumor effects, we set up *in vivo* assays aimed at demonstrating that CTL priming induced by dying tumor cells (as a vaccine) is selectively affected by the loss-of-function mutation of TLR4. These experiments were performed using EG7 cells, a thymoma cell line stably transfected with OVA. Oxaliplatin-treated EG7 cells but not live EG7 cells could elicit the differentiation of OVA-specific IFNγ-producing T cells in the draining lymph node by day five post-vaccine in WT mice. However, Tlr4$^{-/-}$ mice were severely compromised in their capacity to mount an immune response against dying EG7 cells (25). This result was corroborated in C3H/HeJ mice (*Fig. 2A*), which present a naturally occurring defect in the TLR4 signaling pathway. We extended this observation to distinct apoptosis inducers (i.e. using X-rays) (*Fig. 2B*) and tumor antigens. Doxorubicin-treated CT26 colon cancer or MCA205 sarcoma could efficiently prime tumor-specific T lymphocytes in BALB/c and C57BL/6 mice, respectively, if such mice were on a WT or *Tlr2*$^{-/-}$ background. However, no T-cell priming was achieved in *Tlr4*$^{-/-}$ littermates (25). Cross-presentation of OVA from dying EG7 (H-2^{b}) cells was also compromised by the TLR4 defect in hosts carrying a different major histocompatibility complex (MHC) class I allele (H-2^{d}) (25). To formally establish that TLR4 must be harbored by host DCs, we used mice that were transgenic for the diphtheria toxin receptor under the control of the CD11c promoter (29). The TLR4-dependent OVA-specific T-cell immunity was abolished when conventional DCs were ablated by the injection of diphtheria toxin (25).

We next set up *in vitro* experiments to directly assess the role of TLR4 presented by DCs in the triggering of MHC class I and II-restricted T-cell hybridomas (the OVA-specific B3Z and B09710, respectively). In line with the results obtained *in vivo*, live EG7 cells could not be processed and presented by DCs to either of the two hybridomas. Only dying tumor cells (X-ray- or oxaliplatin-treated EG7) could stimulate OVA-specific MHC class I and II-restricted T cells, provided that DCs were derived from WT mice or from animals lacking TLR1, TLR2, TLR3, TLR5, TLR6, TLR7, or TLR9. Only *Tlr4*$^{-/-}$ DCs were deficient in their capacity to present antigen from dying tumor cells *in vitro*. Once again, both *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ (not *Trif*$^{-/-}$) DCs failed to cross-present antigen from dying tumor cells to T cells *in vitro* (25) (*Fig. 3*). Altogether, these data indicate that the TLR4/MyD88 pathway determines the immunogenicity of chemotherapy- or radiotherapy-induced tumor cell death and is required for optimal therapeutic responses of tumors treated by chemotherapy or radiotherapy.

HMGB1 as a ligand for TLR4

Cellular injury, degradation of extracellular matrix, and atherosclerotic lesions can release DAMPs (10, 18). In contrast to most TLRs, which interact with a restricted panel of ligands, TLR2 and TLR4 are promiscuous receptors and can be triggered by a variety of endogenous DAMPs (18). Indeed, a number of endogenous proteins bind and stimulate TLR4, including HSP60, HSP70, oxidized low-density lipoprotein, surfactant protein A, hyaluronan breakdown products (19), fibronectin, β-defensin (30), and the alarmin high-mobility group box 1 protein (HMGB1) (31, 32). The term 'alarmin,' coined by Oppenheim, denotes an array of structurally diverse host proteins rapidly released during infection or tissue damage that have mobilizing and activating effects for host defense and tissue repair. Alarmins include the defensins, eosinophil-derived neurotoxin, cathelicidins, and HMGB1 (33). HMGB1 is a highly mobile nuclear protein (non-histone chromatin-binding protein) that influences transcription and other nuclear transactions (34, 35). HMGB1 is either secreted

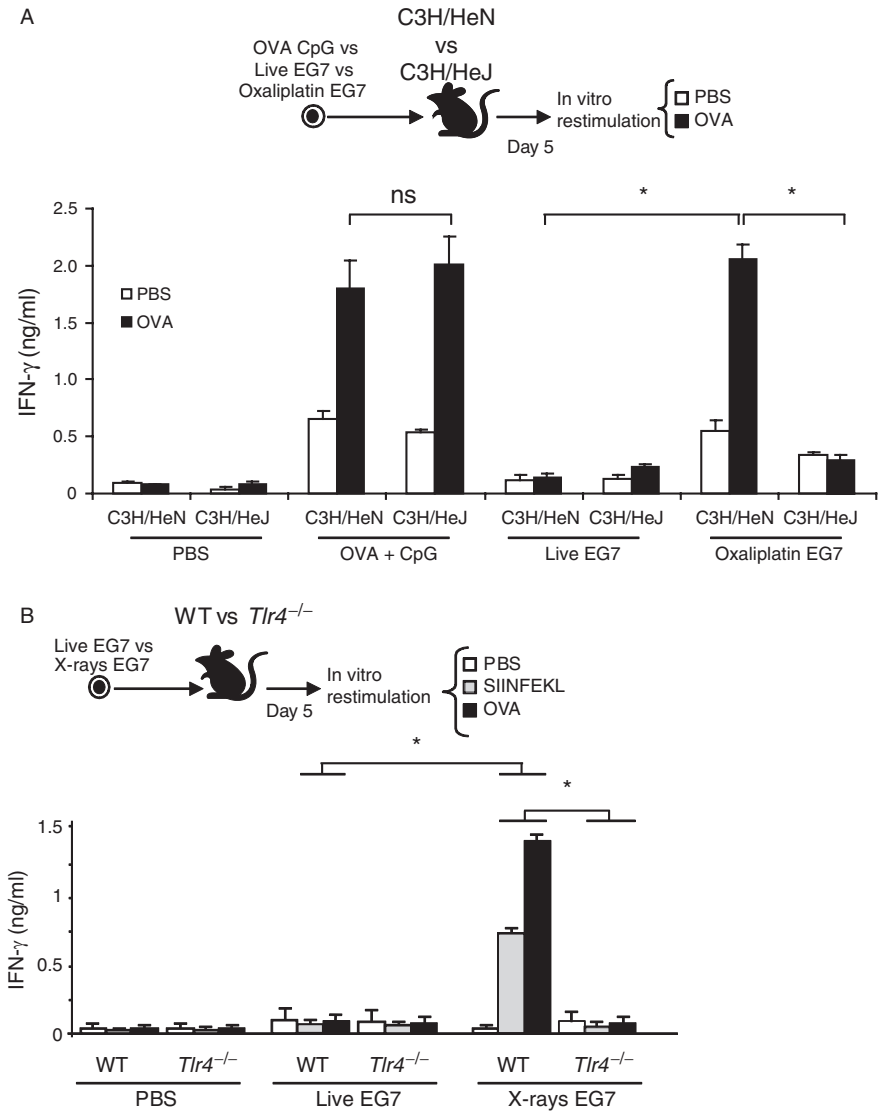

***Fig. 2.* Toll-like receptor 4 (TLR4) signaling is mandatory for the immune response against dying tumor cells *in vivo*.** (A) Dying tumor cells did not elicit an ovalbumin (OVA)-specific immune response in C3H/HeJ mice. Live or oxaliplatin-treated EG7 cells were injected into the footpad of C3H/HeN or C3H/HeJ mice, which harbor a defective TLR4 molecule. As a positive control of antigen presentation, mice were injected with 1 mg of OVA protein plus 10 μg CpG 28 as an adjuvant. Five days later, popliteal lymph node cells were harvested and restimulated with the OVA holoprotein for three days before quantification of interferon (IFN)-γ secretion (B) Dying tumor cells failed to elicit an OVA-specific immune response in $Tlr4^{-/-}$ hosts. Live or X-ray-irradiated EG7 cells were injected into the footpad of C57Bl/6 mice (wildtype or $Tlr4^{-/-}$). Five days later, the local immune response was measured as IFN-γ secretion (as in A). Results (means of triplicates ± SEM, $n = 3$) are typical for three independent experiments. $^{*}P < 0.01$.

actively from inflammatory cells (36) or passively released as a soluble molecule from necrotic cells (37) to signal tissue injury and initiate inflammatory responses through binding to receptor for advanced glycation end products (RAGE), TLR2, or TLR4 (31, 38, 39). It is a terminal mediator of sepsis (40) and an earlier inducer of hepatic injury during ischemia/reperfusion in a TLR4-dependent manner (32).

Irradiation of EG7 and TS/A cells or doxorubicin treatment of CT26 and MCA205 cells caused the late release of HMGB1 (by 18 h) and yet failed to provoke the release or surface exposure of HSPs, β-defensin 2, or fibronectin (25). HMGB1 secretion into the supernatant of dying cells was associated with the reduction of its nuclear expression and its appearance in chromatin-free vesicles (*Fig. 4A*). Acetylcholine and the cholinergic agonist nicotine, by signaling through the α7 nicotinic acetylcholine receptor, inhibited the release of HMGB1 from human and mouse macrophages in response to TNFα or endotoxin (40). We showed that the treatment of tumor cells with X-rays and nicotine could inhibit HMGB1 release from dying tumor cells (*Fig. 4A, B*). Z-VAD-fmk, which suppressed apoptotic caspase activation and delayed secondary necrosis, could also prevent the exodus of HMGB1 from the

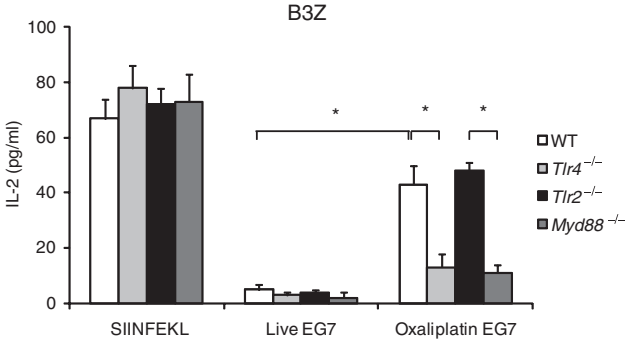

Fig. 3. Toll-like receptor 4 controls antigen presentation by dendritic cells (DCs) engulfing apoptotic bodies in vitro. Bone marrow-derived DCs from C57BL/6 WT, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, or *Myd88*$^{-/-}$ mice were pulsed with the ovalbumin-derived SIINFEKL peptide, live or oxaliplatin-treated EG7 cells (ratio of EG7 to DC 1:1), and were incubated with the SIINFEKL-specific B3Z hybridoma. The ELISA determination of the interleukin-2 concentrations released in the supernatants during the coculture was performed at 48 h. Results (means of triplicates ± SEM, n = 3) are typical for three independent experiments. *P < 0.05.

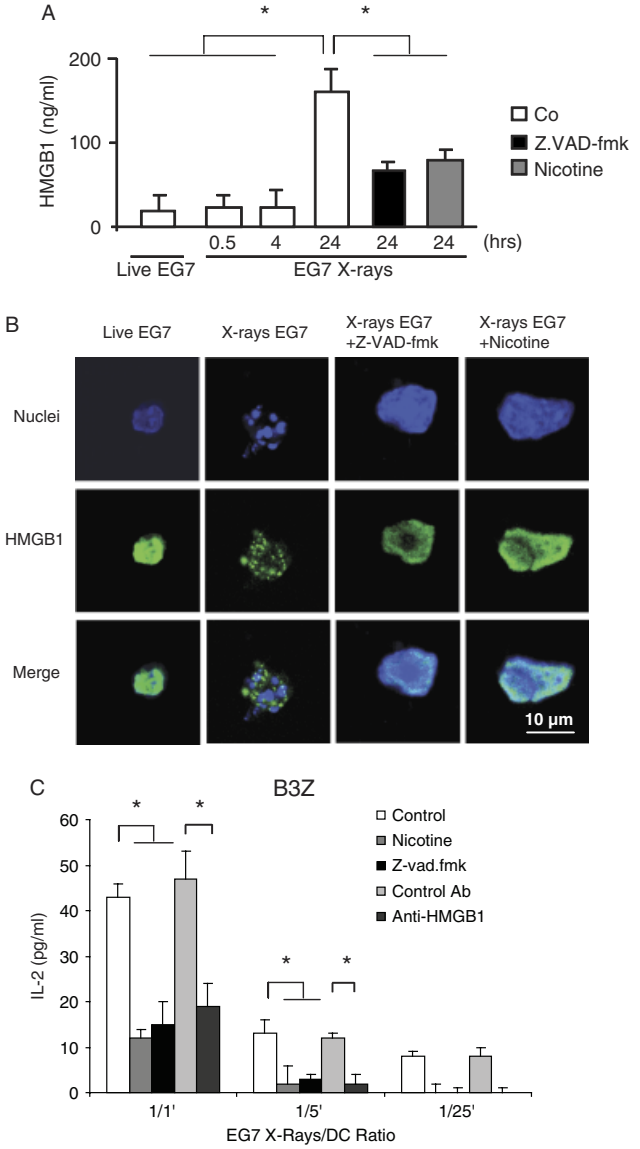

Fig. 4. High mobility group box 1 protein (HMGB1) release by dying tumor cells is required for antigen presentation. (A) Immunoenzymatic detection of HMGB1 in the supernatant of EG7 cells irradiated in the absence or presence of Z-VAD-fmk or nicotine. (B) Confocal immunofluorescence micrographs of live or irradiated EG7 cells, treated in the absence or presence of Z-VAD-fmk or nicotine for 24 h. EG7 cells were stained with the rabbit anti-HMGB1 antibody (or an isotype-matched control antibody) and were counterstained with the goat anti-rabbit Alexa 488. Nuclei were stained with 1 µg/ml ToPro. (C) Inhibition of HMGB1 prevents the antigen presentation to T cells. EG7 cells were irradiated in the absence (Control) or presence of nicotine or Z-VAD-fmk, were washed after 24 h, were loaded onto bone marrow-derived dendritic cells (DCs), and were used to stimulate B3Z T-cell hybridomas. Alternatively, irradiated EG7 cells were loaded onto DCs in the presence of either control antibody or purified rabbit anti-HMGB1 antibody (10 µg/ml). Results (means of triplicates ± SEM, n = 3) are typical for three independent experiments. *P < 0.01.

nucleus (*Fig. 4A, B*). Interference with HMGB1 release, using either z-VAD-fmk or nicotine, severely impaired the immunogenicity of cell death in antigen-presentation assays (*Fig. 3C*). Similar results were obtained using small interfering RNA (siRNA) targeting HMGB1 or a neutralizing anti-HMGB1 antibody, which completely abrogated the immunogenicity of dying tumor cells in all settings (25). Because HMGB1 is involved in the inflammatory response elicited by dying cells (32, 37, 40, 41), we further investigated its contribution to the TLR4-dependent anti-tumor effects. Transfection of doxorubicin-treated CT26 or MCA205 cells with two independent specific siRNAs inhibiting HMGB1 failed to immunize the hosts against a lethal challenge with live tumor cells (25). As expected from earlier reports (31, 38), we could confirm the effective binding of HMGB1 to TLR4 in Raw264.7 macrophages (which express TLR4), using immunoprecipitation with an anti-HMGB1 antibody and blotting with an anti-TLR4 antibody (25). This result supports the contention that HMGB1 secreted by dying tumor cells binds to TLR4 and hence makes it unlikely that another (known or unknown) TLR4 ligand produced by agonizing tumor cells would preferentially occupy TLR4 on antigen-presenting cells. In conclusion, HMGB1 represents (one of) the main DAMPs that dictates the TLR4-dependent immune response against dying tumor cells.

Putative role of TLR4 in DCs

We demonstrated that the interaction between HMGB1 and TLR4 was not required for the phagocytosis of dying tumor cells by DCs or for DC activation (as defined by enhanced membrane expression of MHC class II and costimulatory molecules as well as secretion of TNFα, IL-6, and IL-12p40). The initial observation that TLR4 can inhibit the lysosome-

dependent degradation of phagosomes (42) suggested that *Tlr4*$^{-/-}$ DCs would degrade dying cells in the lysosomal compartment instead of presenting their antigens (43). This assumption prompted us to assess whether TLR4 could be involved in the processing of exogenous cell-associated antigens. Indeed, the antigen cross-presentation by *Tlr4*$^{-/-}$ DCs could be restored by inhibiting the activity of lysosomes, either with chloroquine (a lysosomotropic alkaline) or bafilomycin A1 (a specific inhibitor of the vacuolar adenosine triphosphatase responsible for lysosomal acidification) (25).

Further corroborating these results, we measured the on-rate exposure of MHC class I/peptide complexes (K^b/SIINFEKL) on the surface of DCs (*Tlr4*$^{-/-}$ or *Tlr4*$^{+/+}$) pulsed with dying OVA-transfected TS/A ($H\text{-}2^d$) using the specific 25D1.16 antibody (44). The results clearly indicated a markedly reduced exposure of K^b/SIINFEKL complexes on *Tlr4*$^{-/-}$ DCs compared with their *Tlr4*$^{+/+}$ counterparts, accounting for their impaired ability to induce T-cell activation (25). Importantly, treatment of *Tlr4*$^{-/-}$ DCs with chloroquine restored the ability of DCs to present K^b/SIINKEKL complexes to normal levels, while addition of an anti-HMGB1 antibody completely abrogated the exposure of K^b/SIINFEKL complexes on WT DCs.

We next examined the possibility of enhanced phagosome-endosome/lysosome fusion in *Tlr4*$^{-/-}$ DCs compared with WT DCs, as demonstrated previously in macrophages (42). Our data indicated a significant acceleration of the colocalization of the phagocytic cargo with lysosomes in TLR4-deficient DCs (25). This finding indicated that in contrast to WT DCs, *Tlr4*$^{-/-}$ DCs degrade the antigenic material from phagocytosed dying tumor cells, via rapid fusion of the phagosome with the lysosome.

TLR4 as a predictive factor of response to anthracyclines

Two cosegregating single nucleotide polymorphisms (SNPs) of the human *TLR4* gene, namely Asp299Gly (*rs4986790*) and Thr399Ile (*rs4986791*), have been correlated with a hyporesponsiveness to inhaled lipopolysaccharide (LPS). Epithelial cells and alveolar macrophages derived from probands with the Asp299Gly allele exhibited a decreased response to LPS stimulation *in vitro*, both in the homozygous and in the heterozygous state (45). Because amino acid residues 299 and 399 are situated within the extracellular domain of TLR4, the impact of these SNPs may be caused by a decreased recognition of LPS (46). The associations of these SNPs with the augmented incidence of septic shock during infections with Gram-negative bacteria have also been reported (47–49). We speculated that the identification of *TLR4* Asp299Gly and Thr399Ile mutations might be important for individual risk assessment of patients treated by chemotherapy.

We first addressed the impact of the two *TLR4* SNPs on the binding of HMGB1 to TLR4. We performed immunoprecipitation experiments after adding recombinant HMGB1 to HeLa cells transfected with the normal (Asp299Asp) or the mutated (Asp299Gly) human *TLR4* cDNA. The binding of HMGB1 to the mutant *TLR4* allele was reduced, as compared with the normal *TLR4* (*Fig.* 5). Importantly, mutant TLR4 also reduced the interaction of endogenous TLR4 with HMGB1 (HeLa cells constitutively express a normal TLR4), suggesting that it acts as a dominant-negative form of TLR4 with respect to the binding capacity to HMGB1 (25).

Then, we addressed the functional relevance of the Asp299Gly *TLR4* SNP on the capacity of human monocyte-derived DCs to cross present antigens from dying tumor cells. As compared with normal DCs, DCs derived from individuals bearing the variant *TLR4* allele exhibited a markedly reduced capacity for cross-presenting the MART-1 tumor antigen from dying melanoma cells to a MART-1-specific CTL clone. This defect in antigen presentation could be restored by addition of chloroquine. These data have been obtained for two mutated individuals (25).

We also investigated the clinical relevance of bearing a variant *TLR4* allele for the response to anthracyclines. We carried out the genotyping of *TLR4* in a cohort of breast cancer patients. *TLR4* Asp299Gly genotypes were detected by a single tube polymerase chain reaction (PCR) based on exonuclease degradation of dual-labeled allele-specific oligonucleotides. A cohort of 280 female breast cancer patients presenting with lymph node involvement and treated by surgery, followed by adjuvant irradiation and anthracycline-based chemotherapy were genotyped for *TLR4* and analyzed in terms of progression-free survival after therapy. The frequency of Asp299Gly and Gly299Gly *TLR4* germline polymorphisms was 17.1% and 0.7%, respectively. 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* (50% of relapse in mutated versus 37.4% in non-mutated patients at 10 years, Log-rank test, $P = 0.03$). Similar results were obtained by analyzing the second missense mutation (Thr399Ile) that co-segregates with the Asp299Gly substitution. To underscore the specificity of our clinical findings, we analyzed the impact of irrelevant SNPs affecting a

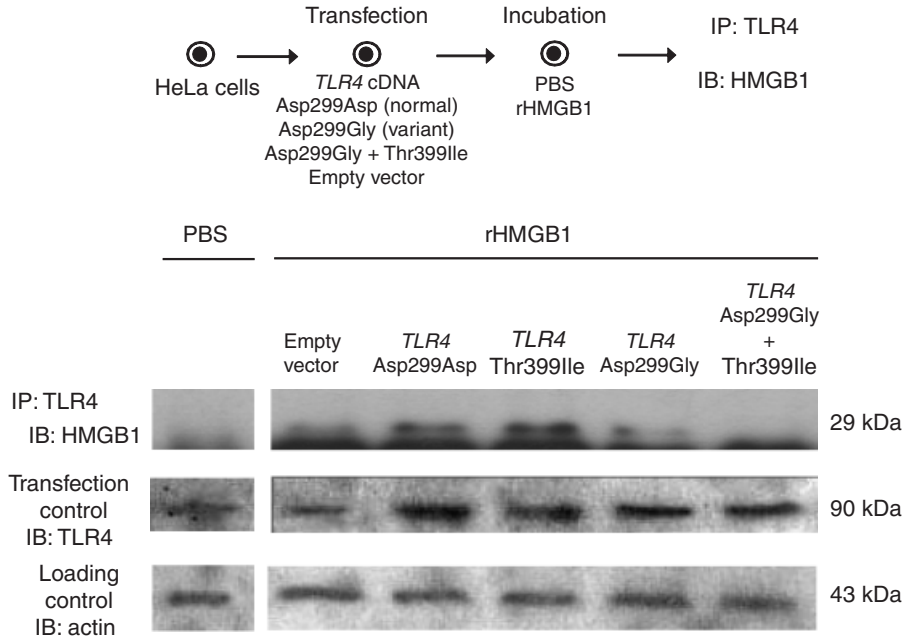

***Fig. 5.* The Asp299Gly and Thr399Ile polymorphisms impair high mobility group box 1 protein (HMGB1) binding to Toll-like receptor 4 (TLR4).** HeLa cells were transfected with a vector containing the *TLR4* Asp299Asp normal cDNA or the *TLR4* Asp299Gly mutated cDNA or an empty vector. Transfectants were incubated in the presence of rHMGB1 for one hour and immunoprecipitation assays using anti-TLR4 antibody (for IP) followed by anti-HMGB1 antibody for blotting (25) were performed as indicated in the scheme.

TLR4 intron and the 5′ untranslated region (*TLR4* mutation rs1927911 and rs10759932, respectively) (50). Moreover, we studied a polymorphism that affects CD14 (which interacts with TLR4 to build up the multi-composite LPS receptor). None of these SNPs influenced the disease-free survival of breast cancer patients (*Fig.* 6). Hence, a mutation of TLR4 that affects its binding to HMGB1 may influence the immunological component of anthracycline-based chemotherapy in human cancer.

Conclusions

The concept of tumor immunosurveillance originally emerged during the 20th century with Burnet's assumption (51) that the immune system participates in the eradication of tumor cells. Critical immune players involved in immunosurveillance, such as T, B, NK, NKT cells, and more recently IFN-producing killer DCs, have been identified (52–56). The findings discussed in this review unravel several novel facets of the concept of tumor immunosurveillance (57).

First, we challenge the view shared by most oncologists that cytotoxic drugs currently used in the clinical armamentarium only act on the tumor cell compartment. Indeed, we demonstrate in four tumor models constrained by three independent therapeutic regimens that cytotoxic compounds also stimulate the host defense against cancer for the optimal control of tumor progression. Second, this cooperation between direct and immune-biased antitumor effects is based on the contention that tumor cell death induced by chemotherapy or ionizing radiation promotes potent tumor-specific cognate immune responses (58, 59). Third, while it was reported that injured or distressed tissues could tune host antigen-presenting cells to elicit T-cell activation (60), our data show that cytotoxic agents could mediate such immunogenic tissue damage. Fourth, the DAMP released by dying tumor cells on chemotherapy or radiotherapy-induced distress has been identified as HMGB1, a nuclear factor that can be released passively during necrosis and actively during late-stage apoptosis. Fifth, while HMGB1 can bind to a variety of different receptors, TLR4 is the only relevant signaling pathway accounting for the immunogenicity of chemotherapy and radiotherapy. Sixth, TLR4 harbored on DCs does not promote DC maturation (accounting for T-cell cross-priming) but rather controls the fusion between phagosomes and lysosomes that causes antigen degradation, deviating the antigen from the processing/presentation compartment. Finally, we provide evidence that the *TLR4* SNP Asp299Gly compromises the binding of HMGB1 to the extracellular domain of the receptor, compromising DC-mediated cross-presentation in patients bearing the mutant allele. The latter experimental finding may explain why this polymorphism predicts a decreased response to anthracyclines in breast cancer patients.

Our data do not reveal all of the mechanisms accounting for the immunogenicity of cell death induced by apoptotic

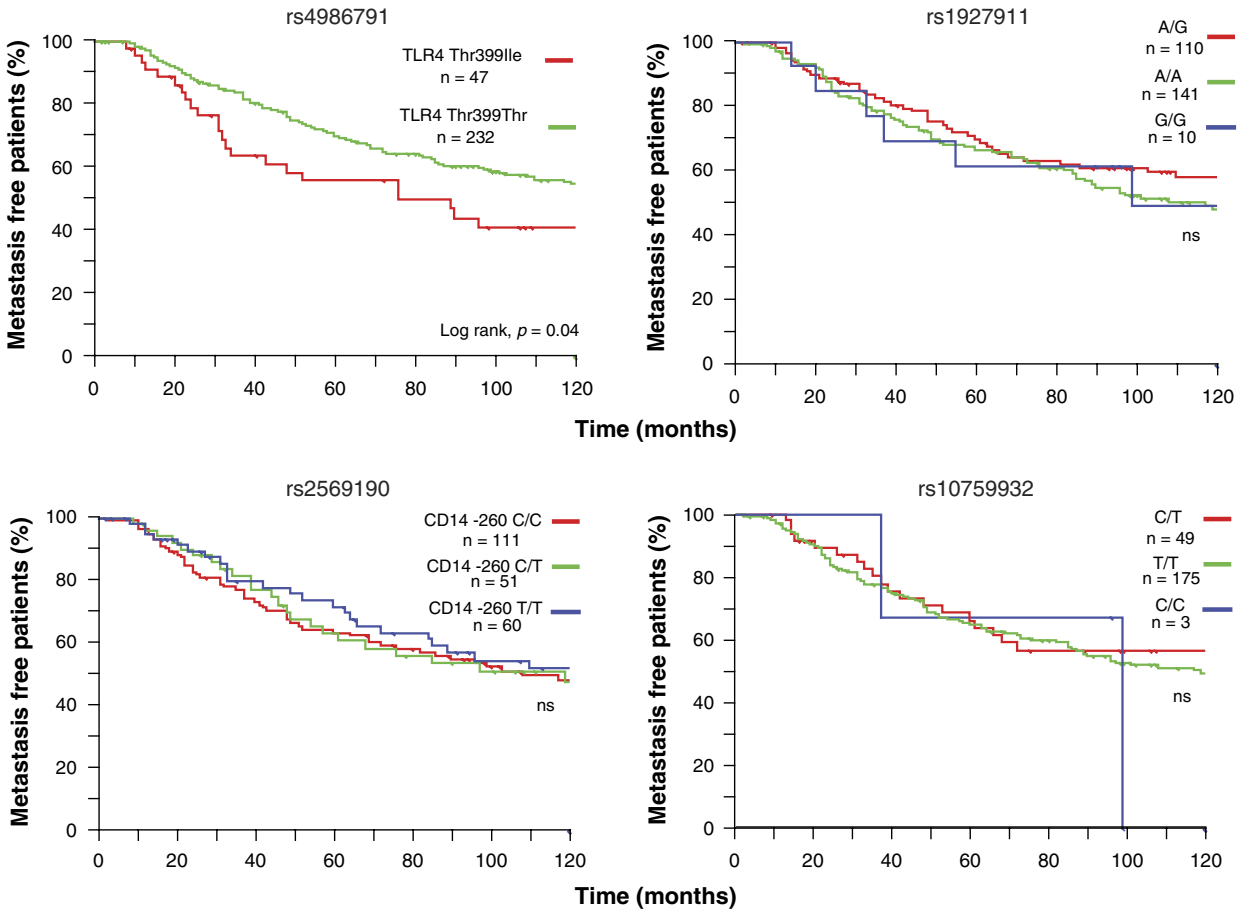

***Fig. 6.* Progression-free survival according to the Toll-like receptor 4 (TLR4) or CD14 status.** Patients were genotyped using Taqman PCR as previously described (80). The Kaplan–Meier estimates of time to metastases among breast cancer patients featuring normal or mutated alleles of TLR4 or CD14 is shown. Note that patients that exhibited a variant *TLR4* allele did not differ from patients displaying the normal *TLR4* allele for all classical prognostic factors. The comprehensive details about the patients' cohort as depicted in Apetoh *et al.* (25).

inducers. Indeed, if ecto-calreticulin is a mandatory eat-me signal (61–63) and HMGB1 is a critical mediator for the TLR4-dependent processing of exogenous tumor antigens by DCs (25), we are still missing a third signal that induces DC maturation. Indeed, recombinant HMGB1 protein, used as a holoprotein, could not promote the alloreactivity of immature bone marrow-derived DCs and monocyte-derived DCs in our hands [although the HMGB1 fragment box B could do so (64)], and the MyD88-dependent maturation of DCs loaded with dying tumor cells was not abrogated by the anti-HMGB1 monoclonal antibody *in vitro* (our unpublished data). Therefore, additional components released or expressed by dying tumor cells need to be identified to delineate the overall pathway of the immunogenicity of cell death. Following from this issue, the question of whether ecto-calreticulin and HMGB1 could also promote the autoreactivity of B and T cells remains open.

The phagosome maturation model (65) proposes that the antigen and the TLR ligand must be present within the same particle to be interpreted by the immune system as 'microbial' or 'dangerous.' Based on this model, it is hard to predict whether soluble HMGB1 would dictate the immunogenicity of cells from the immunological self. It remains to be determined whether (part of) HMGB1 may remain associated with stressed or dying cells or whether other yet-to-be-identified TLR ligands come into play. Moreover, the phagosome maturation model ensures the selection of 'dominant' antigens for presentation by MHC class II molecules; yet, those required for MHC class I presentation remain to be established.

TLR7, TLR8, and TLR9 were originally defined as receptors specific for bacterial and viral RNA (66, 67) or DNA (68). However, more recent studies have provided evidence that these receptors also detect host RNA, DNA, and DNA-associated proteins (69, 70). They probably play a role in the development of systemic autoimmune disorders (71). Tian *et al.* (69) recently demonstrated that class A (but not B) CpG-

containing oligodeoxynucleotides (ODNs) and HMGB1 (this applies both to the holoprotein and to the B-box containing the RAGE-binding domain) functionally interact to stimulate plasmacytoid DCs to produce type 1 IFN and TNFα. CpG-A ODNs augment the binding of HMGB1 to RAGE. HMGB1–DNA complexes resulted in the association of RAGE with TLR9. In addition, HMGB1 was present in the DNA-containing immune complexes and was essential to trigger autoreactive B cells and to induce IFIT1 mRNA, a target gene encoding type 1 IFN (69). Thus, HMGB1 can mediate the activation of TLR9 by DNA-containing immune complexes through a mechanism involving RAGE. How such observations can be reconciled to ours remains elusive. Although we ruled out a role for TLR9 in the immunogenicity of cell death, we cannot exclude a role for RAGE at the present stage. Moreover, it remains possible that HMGB1 might interact with another yet-to-be-discovered molecule that would then physically interact with TLR4. Indeed, Tian *et al.* (69) showed that mammalian HMGB1 failed to bind to a recombinant TLR4-Fc fusion protein, although it did bind to RAGE-Fc *in vitro*, in a cell-free system.

Other cytotoxic agents, including chemotherapy regimen or vascular-disrupting agents (VDA), have been reported to stimulate immune responses and/or to mediate T-cell-dependent anti-tumor effects in mice (72–75). Recently, human *in vitro* studies demonstrated that myeloma cell death induced by bortezomib (but not dexamethasone or irradiation) can be immunogenic, likewise due to the exposure of HSP90 on the surface of bortezomib-treated tumor cells (76). HSP90 was detectable on myeloma plasma membranes by 12 h after treatment and allowed the DC-mediated T-cell cross presentation in the absence of an exogenous maturation stimulus (76).

In addition to offering novel conceptual advances, these data question the validity of certain practices routinely applied in the management of cancer patients. If an HMGB1/TLR4-dependent priming does occur in the draining lymph node of a tumor treated with chemotherapy or radiotherapy, what is the advantage of local lymph node resection? Does the benefit of staging the disease (by lymph node resection) outweigh the abolition of efficient T-cell priming? If the DC-mediated T-cell activation is initiated as a result of chemotherapy- or radiotherapy-induced cell death, what is the risk of prescribing repeated doses of glucocorticoids to combat nausea and vomiting? If dying tumor cells are cross-presented by host DCs, should not we consider to privilege neoadjuvant over adjuvant chemotherapy, i.e. chemotherapy before surgery rather than after surgery? One could predict that the delivery of antigens would be more efficient in the former case, where bulk tumors are treated, rather than in the latter case, where only occult residual disease is targeted. Lastly, we should further explore the functional relevance of TLR4 in the success of chemotherapy. Around 12% of Caucasians carry the 'defective' Asp299Gly *TLR4* allele, and a yet-to-be-determined proportion of individuals present with TLR4 defects at the transcriptional level. A previous report claimed that monocyte-derived DCs generated from two of 10 patients bearing a head and neck tumor profoundly downregulated the expression of normal TLR4 receptors (77). Therefore, manipulations aimed at normalizing functional TLR4 defects may improve the efficacy of chemotherapy in these patients. Although lysosomotropic drugs have been used in a variety of indications including tumors that overexpress the multidrug resistance (MDR) pump (78, 79), chloroquine synergized with oxaliplatin (which is not a

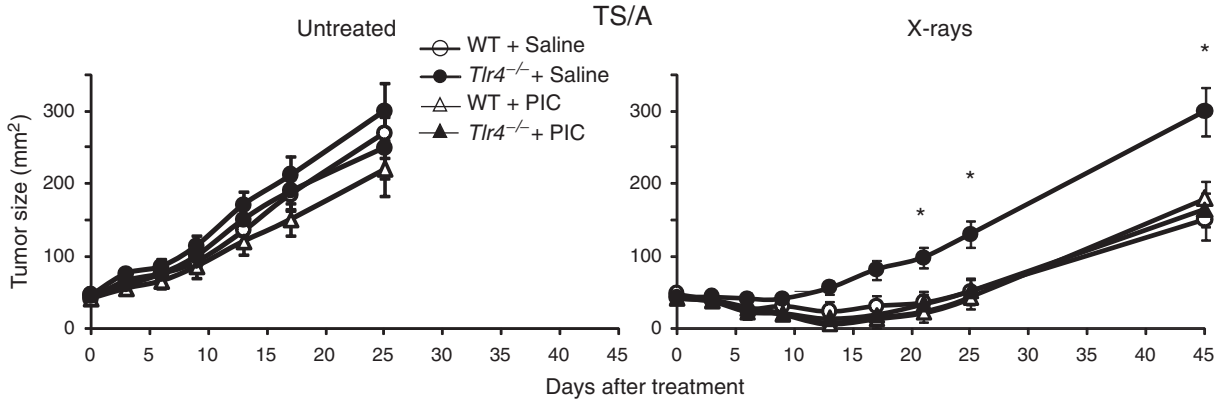

***Fig. 7. Tlr4*$^{-/-}$ mice recover full sensitivity to X-rays in the presence of TLR3 ligands.** The TS/A mammary tumor was inoculated in immunocompetent or *Tlr4*$^{-/-}$ Balb/c mice and irradiated at 10 Grays in one shot when reaching a size of 30–50 mm^2 (around day 9–12). Concomitant therapy with either saline or 50 μg of poly I:C was administered intraperitoneally at days 1, 4, and 7 after radiotherapy into TS/A bearing-animals (left panel) receiving also radiotherapy (right panel). Tumor size was monitored twice a week. Each experiment included five mice per group and was performed twice yielding similar results. *$P < 0.05$.

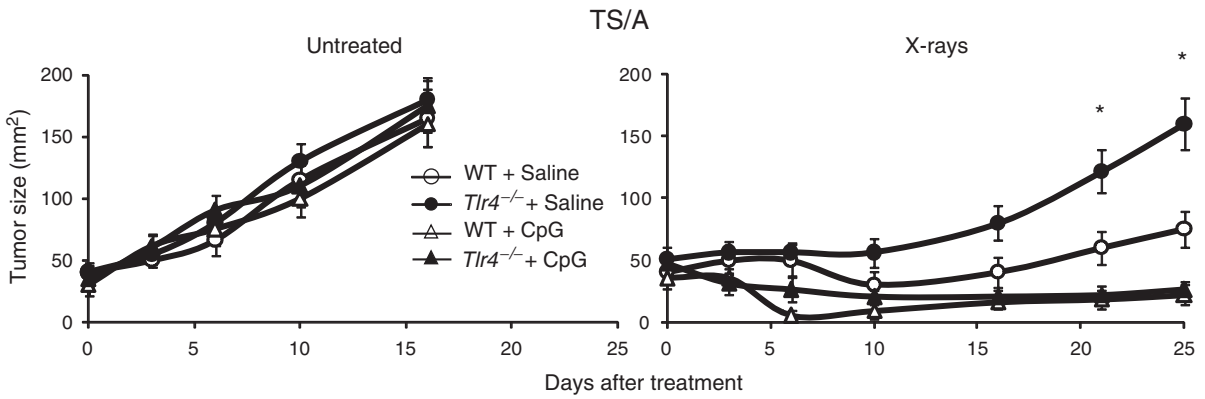

***Fig. 8. Tlr4*$^{-/-}$ mice recover full sensitivity to X-rays in the presence of TLR9 ligands.** Similar experimental setting as in *Fig.* 7, but instead of poly I:C, animals received 50 μg of CpG ODN 28 5′-TCCATGACGTTCCTGACGTT-3′.

substrate of MDR) in *Tlr4*$^{-/-}$ mice (25). Moreover, chloroquine corrected deficient cross-presentation by *TLR4*-mutated mouse bone marrow-derived DCs or human monocyte-derived DCs (25). This finding underscores the potential of combining chloroquine with cytotoxic agents for the treatment of cancer-bearing TLR4-deficient hosts. However, our unpublished data also point to the potential of TLR3 and TLR9 ligands in compensating for TLR4 deficiencies in anti-tumor effects, depending on ionizing radiation (*Figs 7 and 8*). Should the predictive role of the Asp299Gly *TLR4* SNP for the response to anthracyclines be confirmed in breast cancer patients, the challenge will remain to demonstrate prospectively that the combination of doxorubicine and chloroquine is beneficial in patients bearing the mutated allele but not in the cohort carrying the normal allele, independently of MDR.

References

1. Chabner BA, Roberts TG Jr. Timeline: chemotherapy and the war on cancer. Nat Rev Cancer 2005;**5**:65–72.
2. Kanzler H, Barrat FJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with toll-like receptor agonists and antagonists. Nat Med 2007;**13**: 552–559.
3. Phan GQ, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci USA 2003;**100**:8372–8377.
4. Hirano F, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer Res 2005;**65**:1089–1096.
5. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat Rev Immunol 2005;**5**:296–306.
6. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;**392**:245–252.
7. Janeway CA Jr. How the immune system works to protect the host from infection: a personal view. Proc Natl Acad Sci USA 2001;**98**:7461–7468.
8. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila toll protein signals activation of adaptive immunity. Nature 1997;**388**:394–397.
9. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997;**91**:295–298.
10. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 1994;**12**:991–1045.
11. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nat Med 1999;**5**:1249–1255.
12. Kurts C, Miller JF, Subramaniam RM, Carbone FR, Heath WR. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. J Exp Med 1998;**188**:409–414.
13. Shi Y, Rock KL. Cell death releases endogenous adjuvants that selectively enhance immune surveillance of particulate antigens. Eur J Immunol 2002;**32**: 155–162.
14. Tabeta K, et al. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol 2006;**7**:156–164.
15. Janssen E, et al. Efficient T cell activation via a toll-interleukin 1 receptor-independent pathway. Immunity 2006;**24**:787–799.
16. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 2003;**425**:516–521.
17. la Sala A, Ferrari D, Corinti S, Cavani A, Di Virgilio F, Girolomoni G. Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. J Immunol 2001;**166**:1611–1617.
18. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. Nat Rev Immunol 2006;**6**:823–835.
19. Jiang D, et al. Regulation of lung injury and repair by toll-like receptors and hyaluronan. Nat Med 2005;**11**:1173–1179.
20. Zubrod CG. The national program for cancer chemotherapy. JAMA 1972;**222**:1161–1162.
21. Johnson J, Monks A, Hollingshead M, Sausville E In: Chabner BA, Longo D, eds. Cancer Chemotherapy and Biotherapy. Philadelphia: Lippincott Williams & Wilkins, 2001:17–36.
22. Takimoto CH. Anticancer drug development at the US National Cancer Institute. Cancer Chemother Pharmacol 2003;**52** (Suppl.):S29–S33.
23. Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. Eur J Cancer 2004;**40**:827–836.
24. Casares N, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. J Exp Med 2005;**202**:1691–1701.

25. Apetoh L, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 2007;**13**:1050–1059.
26. Granda TG, et al. Circadian optimisation of irinotecan and oxaliplatin efficacy in mice with Glasgow osteosarcoma. Br J Cancer 2002;**86**:999–1005.
27. Bauer AK, et al. Toll-like receptor 4 in butylated hydroxytoluene-induced mouse pulmonary inflammation and tumorigenesis. J Natl Cancer Inst 2005;**97**:1778–1781.
28. Beutler B, et al. Genetic analysis of host resistance: toll-like receptor signaling and immunity at large. Annu Rev Immunol 2006;**24**:353–389.
29. Jung S, et al. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity 2002;**17**:211–220.
30. Biragyn A, et al. Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. Science 2002;**298**:1025–1029.
31. Park JS, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol 2006;**290**:C917–C924.
32. Tsung A, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 2005;**201**:1135–1143.
33. Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ. High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. J Leukoc Biol 2007;**81**:59–66.
34. Bianchi ME, Beltrame M, Paonessa G. Specific recognition of cruciform DNA by nuclear protein HMG1. Science 1989;**243**:1056–1059.
35. Einck L, Bustin M. The intracellular distribution and function of the high mobility group chromosomal proteins. Exp Cell Res 1985;**156**:295–310.
36. Andersson U, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med 2000;**192**:565–570.
37. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;**418**:191–195.
38. Park JS, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 2004;**279**:7370–7377.
39. Dumitriu IE, Baruah P, Bianchi ME, Manfredi AA, Rovere-Querini P. Requirement of HMGB1 and RAGE for the maturation of human plasmacytoid dendritic cells. Eur J Immunol 2005;**35**:2184–2190.
40. Wang H, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999;**285**:248–251.
41. Rovere-Querini P, et al. HMGB1 is an endogenous immune adjuvant released by necrotic cells. EMBO Rep 2004;**5**:825–830.
42. Shiratsuchi A, Watanabe I, Takeuchi O, Akira S, Nakanishi Y. Inhibitory effect of Toll-like receptor 4 on fusion between phagosomes and endosomes/lysosomes in macrophages. J Immunol 2004;**172**:2039–2047.
43. Delamarre L, Couture R, Mellman I, Trombetta ES. Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. J Exp Med 2006;**203**:2049–2055.
44. Porgador A, Yewdell JW, Deng Y, Bennink JR, Germain RN. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. Immunity 1997;**6**:715–726.
45. Arbour NC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;**25**:187–191.
46. Rallabhandi P, et al. Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 stoichiometry, structure, and signaling. J Immunol 2006;**177**:322–332.
47. Agnese DM, et al. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. J Infect Dis 2002;**186**:1522–1525.
48. Schroder NW, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis 2005;**5**: 156–164.
49. Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Arch Intern Med 2002;**162**:1028–1032.
50. Cheng I, Plummer SJ, Casey G, Witte JS. Toll-like receptor 4 genetic variation and advanced prostate cancer risk. Cancer Epidemiol Biomarkers Prev 2007;**16**:352–355.
51. Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J 1957;**1**:779–786.
52. Shankaran V, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001;**410**:1107–1111.
53. Borg C, et al. Novel mode of action of c-kit tyrosine kinase inhibitors leading to NK cell-dependent antitumor effects. J Clin Invest 2004;**114**:379–388.
54. Crowe NY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. J Exp Med 2002;**196**:119–127.
55. Taieb J, et al. A novel dendritic cell subset involved in tumor immunosurveillance. Nat Med **12**:214–219.
56. Ullrich E, et al. Therapy-induced tumor immunosurveillance involves IFN-producing killer dendritic cells. Cancer Res 2007;**67**:851–853.
57. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 2006;**6**:715–727.
58. Galluzzi L, et al. Cell death modalities: classification and pathophysiological implications. Cell Death Differ 2007;**14**:1237–1243.
59. Zitvogel L, Casares N, Pequignot MO, Chaput N, Albert ML, Kroemer G. Immune response against dying tumor cells. Adv Immunol 2004;**84**:131–179.
60. Ghiringhelli F, Apetoh L, Housseau F, Kroemer G, Zitvogel L. Links between innate and cognate tumor immunity. Curr Opin Immunol 2007;**19**:224–231.
61. Obeid M, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 2007;**13**:54–61.
62. Obeid M, et al. Calreticulin exposure is required for the immunogenicity of gamma-irradiation and UVC light-induced apoptosis. Cell Death Differ 2007; Jul 27.
63. Obeid M, et al. Leveraging the immune system during chemotherapy: moving calreticulin to the cell surface converts apoptotic death from 'silent' to immunogenic. Cancer Res 2007;**67**: 7941–7944.
64. Messmer D, et al. High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization. J Immunol 2004;**173**:307–313.
65. Blander JM, Medzhitov R. On regulation of phagosome maturation and antigen presentation. Nat Immunol 2006;**7**:1029–1035.
66. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004;**303**:1529–1531.
67. Heil F, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;**303**:1526–1529.
68. Hemmi H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;**408**:740–745.
69. Tian J, et al. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. Nat Immunol 2007;**8**:487–496.
70. Ivanov S, et al. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. Blood 2007;**110**:1970–1981.

71. Marshak-Rothstein A, Rifkin IR. Immunologically active autoantigens: the role of toll-like receptors in the development of chronic inflammatory disease. Annu Rev Immunol 2007;**25**:419–441.
72. Lake RA, Robinson BW. Immunotherapy and chemotherapy – a practical partnership. Nat Rev Cancer 2005;**5**:397–405.
73. Baguley BC. Antivascular therapy of cancer: DMXAA. Lancet Oncol 2003;**4**: 141–148.
74. Jassar AS, et al. Activation of tumor-associated macrophages by the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid induces an effective CD8+T-cell-mediated antitumor immune response in murine models of lung cancer and mesothelioma. Cancer Res 2005;**65**:11752–11761.
75. Roberts ZJ, et al. The chemotherapeutic agent DMXAA potently and specifically activates the TBK1-IRF-3 signaling axis. J Exp Med 2007;**204**:1559–1569.
76. Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV. Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. Blood 2007;**109**:4839–4845.
77. Frenzel H, Hoffmann B, Brocks C, Schlenke P, Pries R, Wollenberg B. Toll-like receptor interference in myeloid dendritic cells through head and neck cancer. Anticancer Res 2006;**26**:4409–4413.
78. Genne P, Duchamp O, Solary E, Magnette J, Belon JP, Chauffert B. Cinchonine per os: efficient circumvention of P-glycoprotein-mediated multidrug resistance. Anticancer Drug Des 1995;**10**:103–118.
79. Wattel E, et al. Quinine improves the results of intensive chemotherapy in myelodysplastic syndromes expressing P glycoprotein: results of a randomized study. Br J Haematol 1998;**102**:1015–1024.
80. Van Rijn BB, Roest M, Franx A, Bruinse HW, Voorbij HA. Single step high-throughput determination of Toll-like receptor 4 polymorphisms. J Immunol Methods 2004;**289**:81–87.