Toll-like receptor 4–dependent contribution of the immune system to anticancer chemotherapy and radiotherapy

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Conventional cancer treatments rely on radiotherapy and chemotherapy. Such treatments supposedly mediate their effects via the direct elimination of tumor cells. Here we show that the success of some protocols for anticancer therapy depends on innate and adaptive antitumor immune responses. We describe in both mice and humans a previously unrecognized pathway for the activation of tumor antigen–specific T-cell immunity that involves secretion of the high-mobility-group box 1 (HMGB1) alarmin protein by dying tumor cells and the action of HMGB1 on Toll-like receptor 4 (TLR4) expressed by dendritic cells (DCs). During chemotherapy or radiotherapy, DCs require signaling through TLR4 and its adaptor MyD88 for efficient processing and cross-presentation of antigen from dying tumor cells. Patients with breast cancer who carry a *TLR4* loss-of-function allele relapse more quickly after radiotherapy and chemotherapy than those carrying the normal *TLR4* allele. These results delineate a clinically relevant immunoadjuvant pathway triggered by tumor cell death.

Apoptosis has been believed to be a silent cell death modality that does not trigger innate or adaptive immune responses[1,2]. Nonetheless, programmed cell death contributes to the onset of an adaptive immune response either directly[2,3] or indirectly, during bacterial and viral infection[4,5]. It is generally assumed that cell death can elicit an immune response only if dying cells emit 'eat me' and 'danger' signals that mediate their efficient phagocytosis by DCs and the maturation of DCs, respectively. Depending on the cell death inducer, some types of tumor cell death can also induce an antitumor immune response, and this property can be exploited to break tumor-induced immune tolerance[6]. Thus, tumor cell death induced by anthracyclines or X-rays can promote a DC-mediated cytotoxic T-lymphocyte (CTL) response that

confers permanent antitumor immunity[6]. The obligate immunogenic 'eat me' signal generated by dying tumor cells consists in the exposure of calreticulin on the cell surface[7]. This particular 'eat me' signal is found only on the surface of cells that succumb to immunogenic death and not on that of cells dying in an immunologically silent fashion, indicating that it constitutes the first checkpoint for the immuno-adjuvant effect of tumor cell death[8]. However, calreticulin exposure is not sufficient to elicit an antitumor immune response, because live cells that express ecto-calreticulin are unable to induce DC maturation and antigen presentation and hence are non-immunogenic[8]. This suggests that tumor cells must emit one or several additional signals linked to cell death in order to trigger an efficient immune response.

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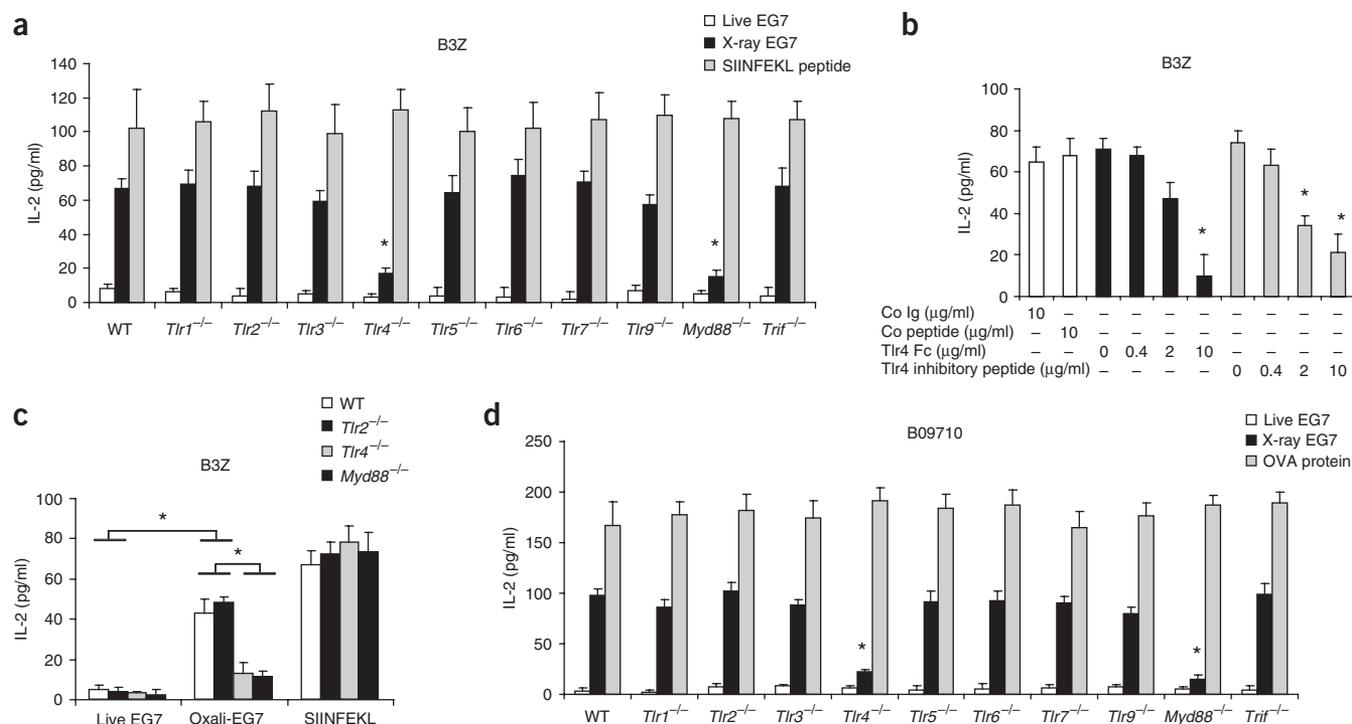
Figure 1 TLR4 controls antigen presentation by DCs engulfing apoptotic bodies *in vitro*. (**a**) TLR4 and MyD88 are required for antigen presentation by DCs loaded with irradiated tumor cells to MHC class I (H-2^b)-restricted T cells. Shown are IL-2 levels in the supernatants from DCs of the indicated genotype (background C57BL/6, H-2^b I-A^b) after they were pulsed with either SIINFEKL peptide or live or irradiated EG7 cells (ratio of EG7 to DCs 1:1) and incubated with the SIINFEKL-specific B3Z hybridoma cells for 48 h. (**b**) Inhibition of TLR4 abrogates antigen presentation. WT DCs were loaded with decreasing amounts of recombinant TLR4-Fc fusion protein (or a control immunoglobulin (Co Ig)) or a TLR4-inhibitory peptide (or a control peptide (Co peptide)), along with irradiated EG7 cells (as in **a**), and assayed for their capacity to elicit IL-2 production by B3Z cells. (**c**) Same setting as in **a**, using live or oxaliplatin-treated tumor cells. (**d**) TLR4 and MyD88 are required for class II-restricted antigen presentation by DC. Same setting as in **a**, using MHC class-II (I-A^b) restricted hybridoma (B09710). Results (means of triplicates ± s.e.m.) are representative of five experiments, and asterisks indicate significant inhibitory effects of TLR4 inhibition or ablation. *$P < 0.05$.

Toll-like receptors (TLRs) recognize molecules derived from pathogens as well as endogenous danger signals possessing similar chemical structures[9,10]. Upon recognition of their ligands, TLRs transduce signals through two pathways involving distinct adaptors, Toll/IL-1R domain–containing adaptor inducing IFNα (TRIF) and myeloid differentiation primary response protein (MyD88), which is used by all TLRs except TLR3. During microbial infections in which cross-presentation of exogenous antigens is a prerequisite for T-cell activation[11], TLRs present on the surface of DCs or macrophages are triggered by alien components and mediate the activation of antigen-presenting cells (APCs)[5,9]. In addition, TLRs present on internal membranes may control the processing and presentation of peptides derived from internalized cargo[12–14].

While searching for the TLRs[15] that might be involved in the immune response against dying tumor cells, we found that TLR4 expression by DCs is a prerequisite for efficient antigen presentation of tumor antigens furnished by dying cancer cells. This observation led us to the discovery of the 'danger' signal emitted by dying tumor cells: the release of the HMGB1 protein. We demonstrate that both the release of HMGB1 by dying tumor cells and the TLR4–myeloid differentiation primary response protein-88 (MyD88) signaling pathway are required for the immune response against dying tumor cells and also for the efficacy of anticancer chemotherapy and radiotherapy in mice. The clinical relevance of these findings is underscored by the observation that the Asp299Gly *TLR4* mutation, which affects the

binding of HMGB1 to the receptor, has a negative prognostic impact on human patients with breast cancer.

RESULTS

TLR4 is required for cross-presentation of dying tumor cells

To determine which TLR might control the immune response against dying tumor cells, we fed dying ovalbumin (OVA)-expressing EG7 mouse thymoma cells to bone marrow–derived DCs (BM-DCs) that were either wild type (WT) or lacking TLRs. We then assessed the antigen-presenting capacity of the DCs by measuring IL-2 production by MHC class I (H-2^b)-restricted OVA$_{257–264}$-specific B3Z and MHC class II (I-A^b)-restricted OVA$_{323–339}$-specific B09710 mouse hybridomas (**Fig. 1**). OVA peptides from irradiated (**Fig. 1a,b,d**) or oxaliplatin-treated (**Fig. 1c**) EG7 tumor cells, but not from live tumor cells, were efficiently presented by DCs. Although syngeneic WT, *Trif*$^{-/-}$, *Tlr1*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$, *Tlr5*$^{-/-}$, *Tlr6*$^{-/-}$, *Tlr7*$^{-/-}$ or *Tlr9*$^{-/-}$ DCs could present antigen from dying tumor cells, *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ DCs were defective in this function (**Fig. 1a,c,d**). Pulsing of DCs from WT mice with a TLR4 inhibitory peptide[16] or a TLR4-Fc fusion protein also inhibited the MHC class I–restricted OVA-specific response (**Fig. 1b**). In contrast, no such inhibitory effect was observed for an oligonucleotide designed to block both TLR7 and TLR9 (data not shown)[17].

Inoculation of oxaliplatin-treated (but not live) EG7 cells into the footpad primed draining lymph node (DLN) cells for interferon-γ

(IFN-γ) production after *in vitro* re-stimulation with OVA protein. This response was obtained in WT and was intact in all *Tlr⁻/⁻* mice except for *Tlr4⁻/⁻* mice (**Fig. 2a**). We confirmed this observation for distinct apoptosis inducers and tumor antigens. Mouse CT26 (H-2^{d+} colon cancer) or MCA205 (H-2^{b+} sarcoma) cells treated with doxorubicin efficiently primed tumor-specific T lymphocytes in BALB/c and C57BL/6 mice with, respectively, a WT or a *Tlr2⁻/⁻* genetic background (**Fig. 2b**). However, neither tumor cell type elicited efficient T-cell priming in *Tlr4⁻/⁻* littermates (**Fig. 2b**). Cross-presentation of OVA from dying EG7 (H-2^b) cells was also compromised by the knockout of *Tlr4* in hosts carrying a different

MHC class I allele (H-2^d) (**Fig. 2c**). Similar results were obtained in C3H/HeJ mice (H-2^k), which are defective in TLR4 signaling (data not shown). In contrast, *Tlr4⁻/⁻* and C3H/HeJ mice mounted an intact response to soluble OVA protein mixed with TLR9 agonists (CpG oligodeoxynucleotides) (**Fig. 2c** and data not shown). Thus, the absence of TLR4 selectively compromised the immune response against dying cells, not soluble antigen.

T-cell priming by dying tumor cells depended stringently on DCs. Injection of diphtheria toxin into mice expressing a transgenic diphtheria toxin receptor (DTR) in DCs[11] led to DC depletion and abrogated the priming of T lymphocytes elicited by dying tumor cells
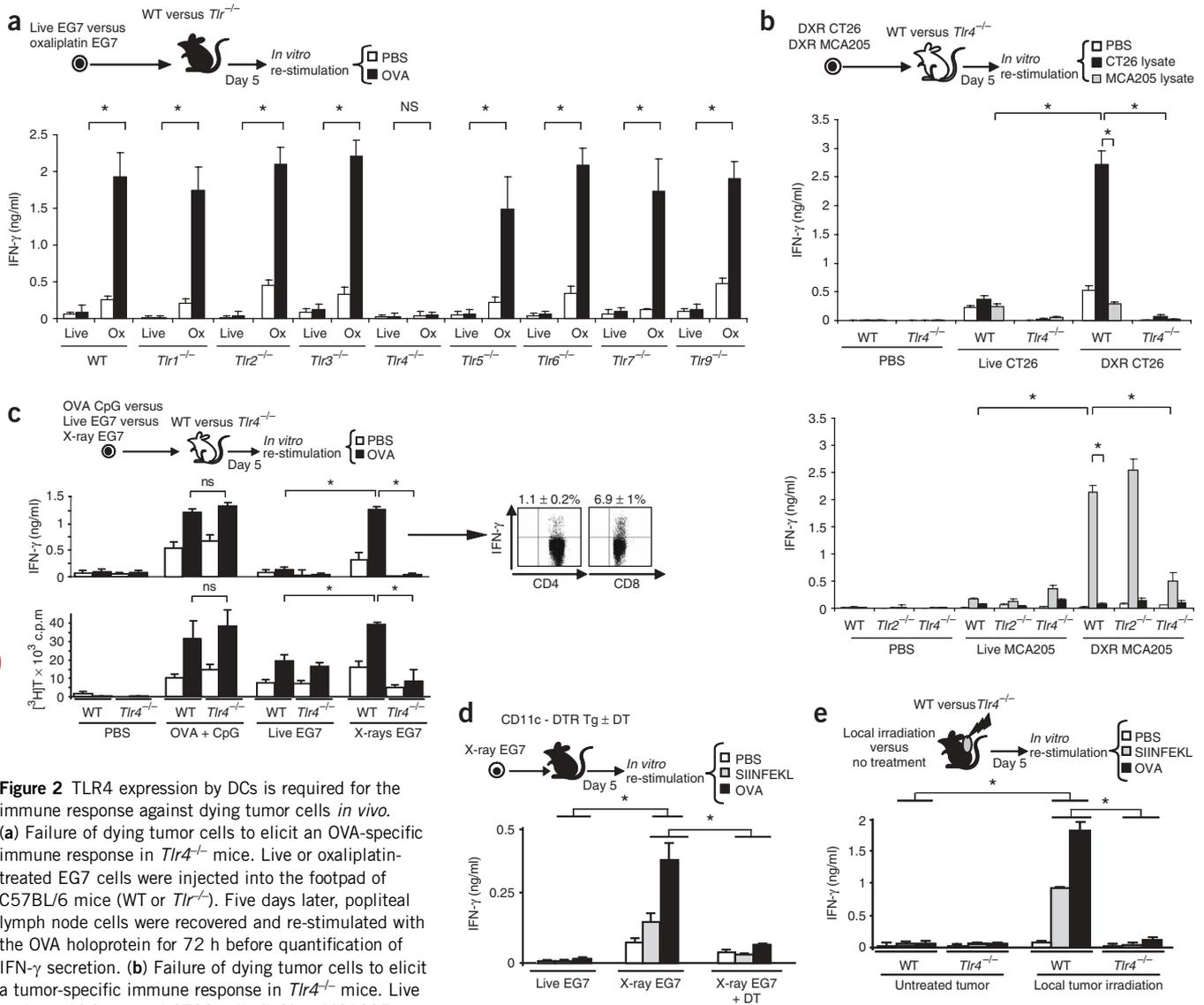
Figure 2 TLR4 expression by DCs is required for the immune response against dying tumor cells *in vivo*. (**a**) Failure of dying tumor cells to elicit an OVA-specific immune response in *Tlr4⁻/⁻* mice. Live or oxaliplatin-treated EG7 cells were injected into the footpad of C57BL/6 mice (WT or *Tlr⁻/⁻*). Five days later, popliteal lymph node cells were recovered and re-stimulated with the OVA holoprotein for 72 h before quantification of IFN-γ secretion. (**b**) Failure of dying tumor cells to elicit a tumor-specific immune response in *Tlr4⁻/⁻* mice. Live or doxorubicin-treated CT26 cells (left) or MCA205 (right) were injected into the footpad of BALB/c or C57BL/6 mice (WT or *Tlr⁻/⁻*), respectively. Lymph node cells were analyzed 5 d later for their capacity to produce IFN-γ upon *in vitro* re-stimulation with tumor lysate. (**c**) Cross-presentation of antigen from dying tumor cells is impaired in *Tlr4⁻/⁻* hosts. Live or irradiated EG7 cells (syngeneic to C57Bl/6 mice) were inoculated into the footpad of WT or *Tlr4⁻/⁻* BALB/c mice, and the local immune response was measured either as IFN-γ secretion (as in **a**) or as proliferation. As a positive control of antigen presentation, mice were injected with 1 mg of OVA protein plus 10 μg CpG 28 as an adjuvant. The inset illustrates that IFN-γ elicited by cross-presentation is produced by both CD4$^+$ and CD8$^+$ T cells. (**d**) Requirement for DCs to mount an immune response against dying tumor cells. Irradiated EG7 cells were injected into C57BL/6 mice expressing a transgenic DTR under the control of the CD11c promoter, and the mice were simultaneously injected i.p. with PBS or diphtheria toxin (DT). The local immune response was assessed 5 d later as in **a**. (**e**) TLR4 is required for the immune response promoted by local tumor radiotherapy *in vivo*. EG7 tumors established in the thigh were X-ray irradiated (10 Gy), and inguinal lymph node cells were analyzed 5 d later for their capacity to produce IFN-γ upon *in vitro* re-stimulation. Results (means of triplicates ± s.e.m. *n* = 3) are representative of a typical experiment out of three independent ones. *$P < 0.01$.

(Fig. 2d). To confirm that it was the TLR4 present in DCs that determined the immune response, we pulsed WT or $Tlr4^{-/-}$ DCs with live or irradiated EG7 cells, transferred them into $Tlr4^{-/-}$ hosts and monitored the priming of CD8^{+} T cells in DLN cells. TLR4 deficiency affecting DCs specifically abolished CTL activation (measured as IFN-γ production and proliferation). However, the absence of TLR4
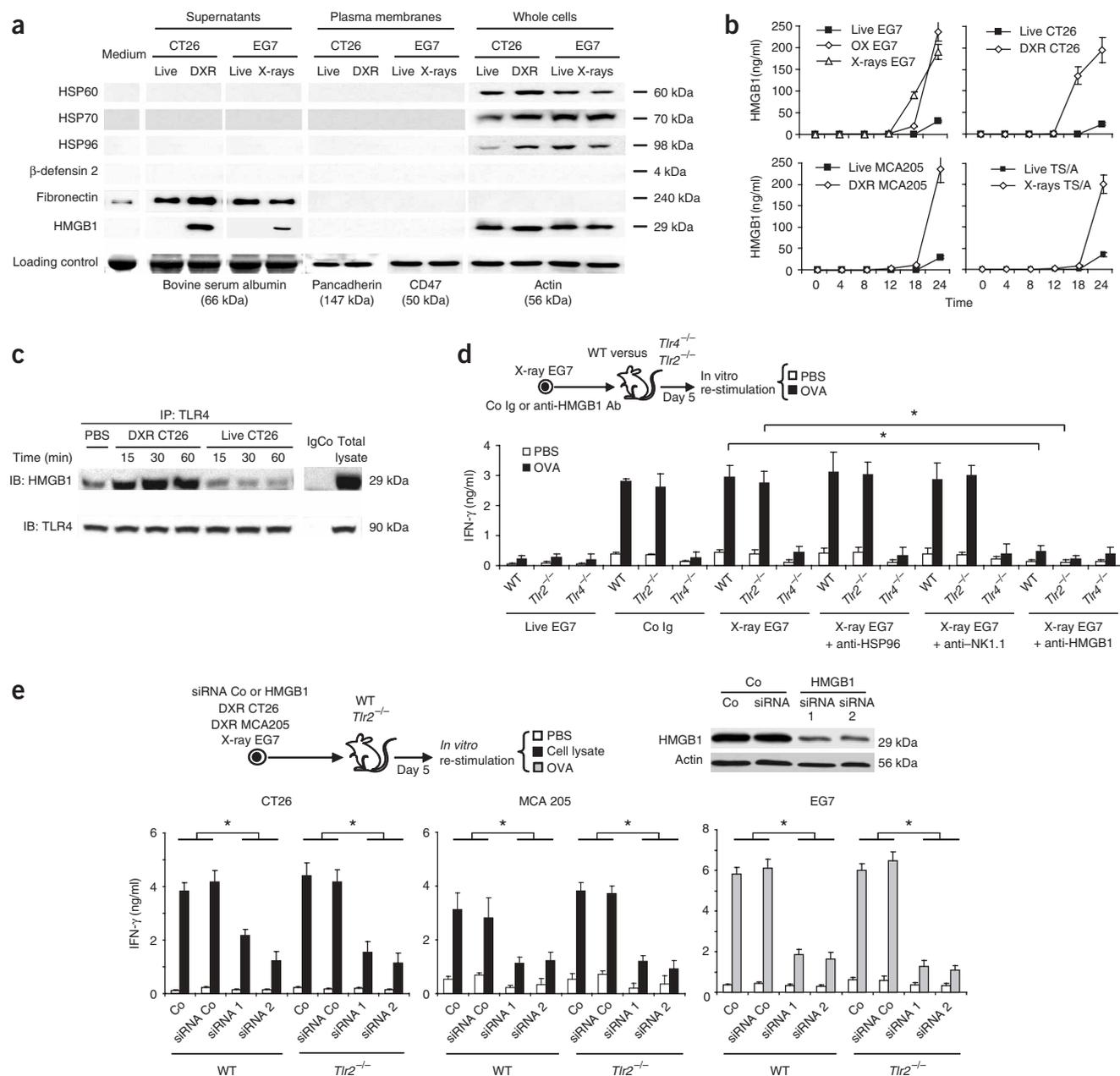
Figure 3 The immunogenicity of dying tumor cells after chemotherapy or radiotherapy depends on the release of the TLR4 ligand HMGB1. (a) HMGB1 is selectively released by dying tumor cells. TLR4 ligands were detected by immunoblots in supernatants, plasma membranes and whole-cell lysates of CT26 and EG7 tumors treated (or not treated) with, respectively, doxorubicin and X-rays. (b) Kinetic study of HMGB1 release from tumor cell lines. Shown is the accumulation of HMGB1, as measured by ELISA, after the indicated treatment of EG7, CT26, MCA205 or TS/A tumor cell lines (c) HMGB1 released from tumor cells binds to TLR4. RAW264.7 cells were incubated with the supernatants of either untreated or doxorubicin-treated CT26 tumor cells for the indicated periods of time. HMGB1 was then detected by western blotting after immunoprecipitation using an antibody against TLR4 (or a control immunoglobulin). The immunoprecipitation assays were performed four times with identical results. (d) Cross-presentation of OVA from dying tumor cells is dependent on HMGB1 in vivo. Live or X-irradiated EG7 were inoculated into the footpads of WT, $Tlr4^{-/-}$ or $Tlr2^{-/-}$ BALB/c mice along with an antibody to HMGB1 antibody (or a control immunoglobulin for each individual antibody (Co Ig) and antibodies to HSP96 and NK1.1 (directed against EG7 membrane-associated antigen)), and the local immune response was measured as in Figure 2a,b. (e) Depletion of HMGB1 in dying tumor cells using siRNA abolished antigen presentation. CT26, MCA205 and EG7 tumor cells were transfected with a control siRNA or two different HMGB1-specific siRNA. After 48 h (when immunoblots confirm HMGB1 depletion, top), the cells were injected into mice as in Figure 2b. Note that HMGB1 depletion does not alter cell death induction by genotoxic stress (data not shown). All in vivo experiments involved three mice per group and were repeated three times yielding identical results. Graphs (means of triplicates ± s.e.m., $n = 3$) are representative of a typical experiment out of three independent ones. *$P < 0.01$.
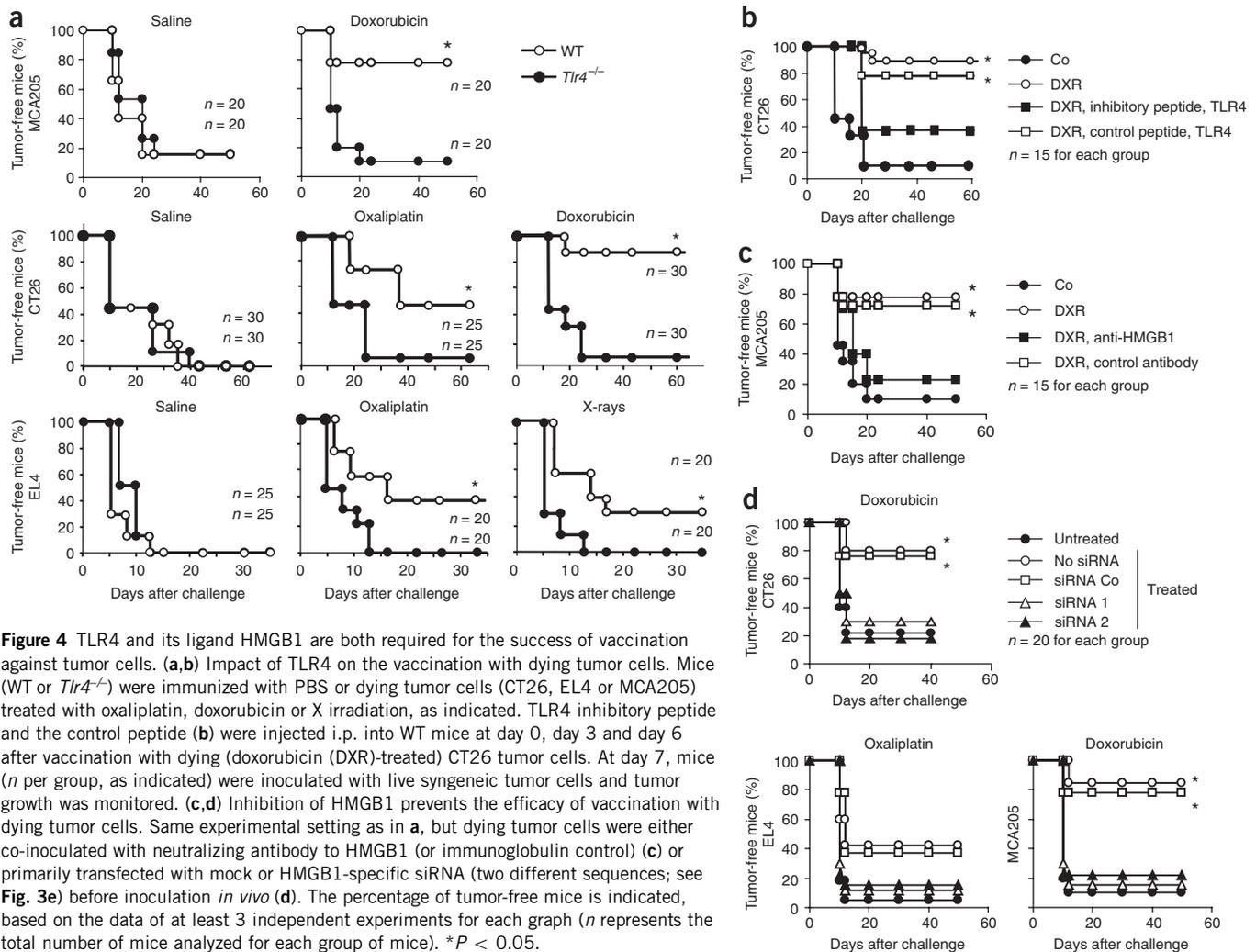
Figure 4 TLR4 and its ligand HMGB1 are both required for the success of vaccination against tumor cells. (**a**,**b**) Impact of TLR4 on the vaccination with dying tumor cells. Mice (WT or $Tlr4^{-/-}$) were immunized with PBS or dying tumor cells (CT26, EL4 or MCA205) treated with oxaliplatin, doxorubicin or X irradiation, as indicated. TLR4 inhibitory peptide and the control peptide (**b**) were injected i.p. into WT mice at day 0, day 3 and day 6 after vaccination with dying (doxorubicin (DXR)-treated) CT26 tumor cells. At day 7, mice (*n* per group, as indicated) were inoculated with live syngeneic tumor cells and tumor growth was monitored. (**c**,**d**) Inhibition of HMGB1 prevents the efficacy of vaccination with dying tumor cells. Same experimental setting as in **a**, but dying tumor cells were either co-inoculated with neutralizing antibody to HMGB1 (or immunoglobulin control) (**c**) or primarily transfected with mock or HMGB1-specific siRNA (two different sequences; see **Fig. 3e**) before inoculation *in vivo* (**d**). The percentage of tumor-free mice is indicated, based on the data of at least 3 independent experiments for each graph (*n* represents the total number of mice analyzed for each group of mice). *$P < 0.05$.

did not impair the capacity of DCs to present the soluble peptide SIINFEKL (**Supplementary Fig. 1** online). In addition, EG7 tumors established in the thigh were irradiated (10 Gy), and this local irradiation stimulated OVA-specific T-cell responses in the inguinal lymph node in WT but not $Tlr4^{-/-}$ mice (**Fig. 2e**). In conclusion, TLR4 must be present in DCs for the optimal presentation of antigen derived from dying tumor cells.

TLR4 controls tumor antigen processing and presentation

WT and $Tlr4^{-/-}$ DCs were equally efficient in engulfing irradiated EG7 thymoma or doxorubicin-treated CT26 colon carcinoma cells (**Supplementary Fig. 2a** online). The acquisition of maturation markers (including MHC class II and the co-stimulatory molecules CD40, CD80, CD86), the production of inflammatory cytokines (IL-6, IL-12p40, TNF-α) and allostimulatory potential by $Tlr4^{-/-}$ DCs were deficient in response to bacterial lipopolysaccharide yet intact in response to dying tumor cells (data not shown and **Supplementary Fig. 2b,c**). However, TLR4 influenced the kinetics at which DCs express K^b-SIINFEKL MHC class I peptide complexes at the plasma membrane surface after loading with dying OVA-transfected TS/A ($H-2^d$) cells. WT and $Tlr4^{-/-}$ DCs expressed comparable levels of K^b molecules at baseline and acquired K^b-SIINFEKL complexes after pulsing with saturable amounts of free SIINFEKL peptides with similar kinetics (data not shown). However, a markedly reduced

exposure of K^b-SIINFEKL complexes was detected on $Tlr4^{-/-}$ DCs, as compared with WT controls, after loading with dying OVA-expressing TS/A cells (**Supplementary Fig. 3a** online).

TLR4 is likely to affect the processing and presentation of antigen. TLR4 has been reported to inhibit the lysosome-dependent degradation of phagosomes[18], meaning that $Tlr4^{-/-}$ DCs would degrade dying cells in the lysosomal compartment instead of presenting their antigens[19]. Indeed, the alkalinization of lysosomes with either chloroquine (a lysosomotropic alkaline) or bafilomycin A1 (a specific inhibitor of the vacuolar ATPase responsible for lysosomal acidification), used at subtoxic concentrations, enhanced the capacity of $Tlr4^{-/-}$ DCs to present antigen from dying cells yet did not ameliorate antigen presentation by WT DCs (**Supplementary Fig. 3b**). Accordingly, treatment of $Tlr4^{-/-}$ DCs with chloroquine restored the ability of DCs to present K^b-SIINKEKL complexes to normal levels (**Supplementary Fig. 3a**, right). Next, we directly determined the kinetics of fusion between phagosomes and lysosomes in WT versus $Tlr4^{-/-}$ DCs loaded with dying tumor cells. Colocalization of the phagocytic cargo with lysosomes was significantly accelerated in $Tlr4^{-/-}$ DCs as compared with WT DCs (**Supplementary Fig. 3c**).

These data confirm that TLR4 regulates the processing and presentation of tumor cell antigens by DCs, presumably by inhibiting the lysosomal destruction of antigens.
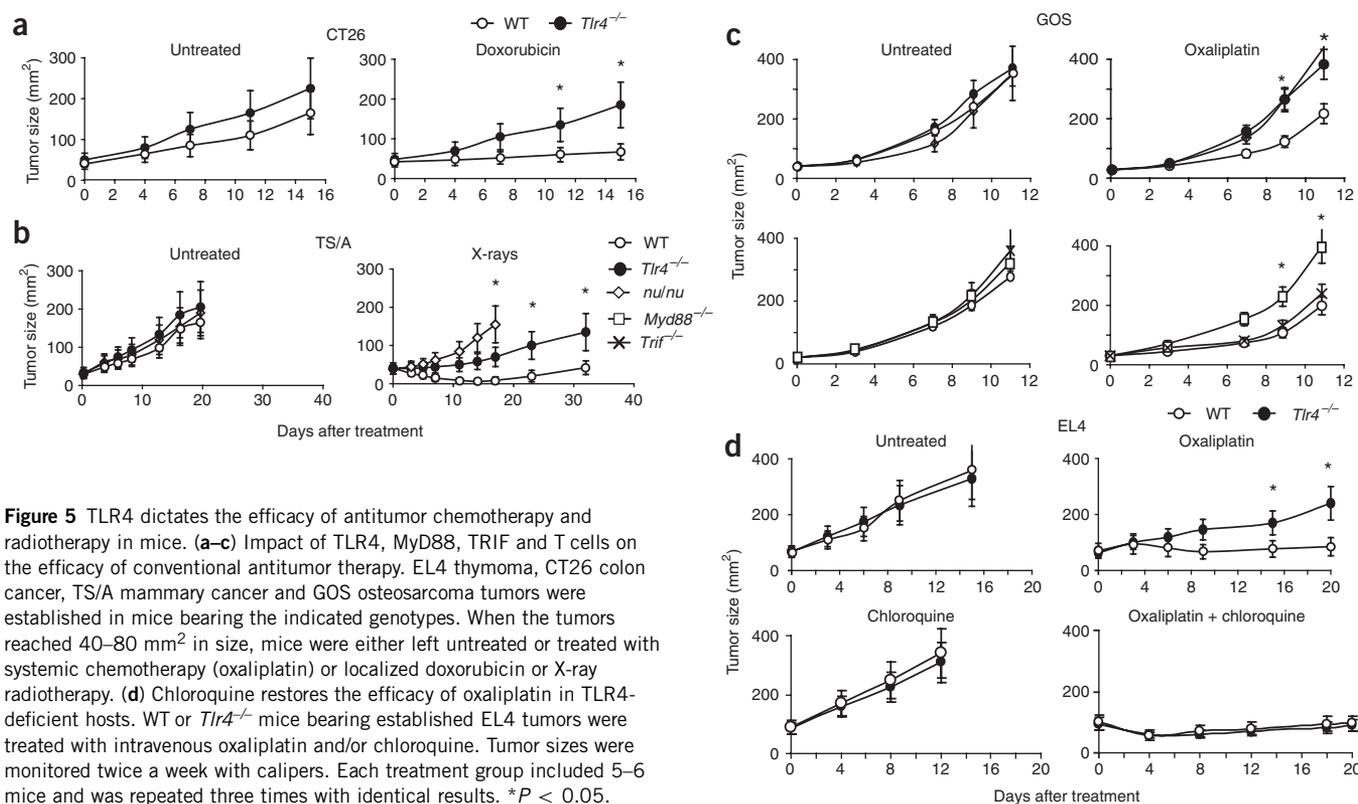
Figure 5 TLR4 dictates the efficacy of antitumor chemotherapy and radiotherapy in mice. (a–c) Impact of TLR4, MyD88, TRIF and T cells on the efficacy of conventional antitumor therapy. EL4 thymoma, CT26 colon cancer, TS/A mammary cancer and GOS osteosarcoma tumors were established in mice bearing the indicated genotypes. When the tumors reached 40–80 mm^2 in size, mice were either left untreated or treated with systemic chemotherapy (oxaliplatin) or localized doxorubicin or X-ray radiotherapy. (d) Chloroquine restores the efficacy of oxaliplatin in TLR4-deficient hosts. WT or Tlr4$^{-/-}$ mice bearing established EL4 tumors were treated with intravenous oxaliplatin and/or chloroquine. Tumor sizes were monitored twice a week with calipers. Each treatment group included 5–6 mice and was repeated three times with identical results. *P < 0.05.

HMGB1 release by dying tumor cells

Reportedly, a number of endogenous proteins bind and stimulate TLR4[20]: heat-shock protein (HSP) 60, HSP70, oxidized LDL, surfactant protein A, hyaluronan breakdown products[21], fibronectin, β-defensin-2 (ref. 22) and the alarmin high-mobility-group box 1 protein (HMGB1)[23,24]. Irradiation of EG7 cells or doxorubicin treatment of CT26 caused the release of HMGB1, yet did not provoke the release or surface exposure of HSPs, β-defensin-2 or fibronectin (**Fig. 3a**). HMGB1 was released 18 h after irradiation of EG7 or TS/A cells or doxorubicin treatment of CT26 or MCA205 cells (**Fig. 3b**), and this release was inhibited by Z-VAD-fmk (data not shown), which suppresses apoptotic caspase activation and delays secondary necrosis. Next we determined whether the HMGB1 contained in the supernatant of dying tumor cells might directly interact with TLR4[23]. Raw264.7 macrophages (which express TLR4) were incubated with supernatants from doxorubicin-treated CT26 cells (containing > 200 ng/ml of free HMGB1) or live CT26 cells (containing < 20 ng/ml of free HMGB1), washed and then subjected to the immunoprecipitation of HMGB1, and TLR4 was detected in the precipitate (**Fig. 3c**). These results demonstrate that HMGB1 secreted by dying tumor cells binds to TLR4 and hence make it unlikely that another (known or unknown) TLR4 ligand contained in this supernatant would preferentially occupy TLR4 on antigen presenting cells.

Inhibition of HMGB1 secretion by preincubation of the tumor cells with a small interfering RNA (**Supplementary Fig. 4** online) inhibited the capacity of irradiated EG7 cells to stimulate B3Z cells via DCs. Similar data were obtained when DCs were cocultured with dying EG7 tumor cells in the presence of neutralizing antibody to HMGB1 (**Supplementary Fig. 4**). Because HMGB1 is involved in the inflammatory response elicited by dying cells[24–27], we further investigated its contribution to the TLR4-dependent antitumor immune response. Neutralization of all possible TLR4 ligands with recombinant TLR4-Fc

fusion protein (**Fig. 1b**) was as efficient in inhibiting the DC-mediated presentation of OVA from irradiated EG7 cells as was neutralization of HMGB1 (**Supplementary Fig. 4**), suggesting that HMGB1 is indeed the principal TLR4-activating agent involved in this system. Local injection of HMGB1-neutralizing antibody (but not an irrelevant antibody to NK1.1, antibody targeting NK1.1 molecules expressed on EG7 or an antibody to HSP96) also inhibited the priming of T cells induced by irradiated EG7 cells (**Fig. 3d**) or doxorubicin-treated CT26 cells (data not shown) in vivo. Similarly, doxorubicin-treated CT26 or MCA205 cells and irradiated EG7 cells lost their capacity to prime T cells in vivo when they were depleted from HMGB1 by means of specific siRNAs (**Fig. 3e**). In conclusion, HMGB1 represents the principal damage-associated molecular pattern that dictates the TLR4-dependent immune response to dying tumor cells.

HMGB1/TLR4/MyD88 in the efficacy of anticancer drugs

Injection of doxorubicin-treated CT26 colon cancer cells is highly efficient in inducing an immune response that prevents the growth of live CT26 cells inoculated 1 week later[6]. We obtained similar results with doxorubicin-treated MCA205 sarcoma cells, which prevented the growth of MCA205. Although this effective vaccination induced by dying tumor cells applied to WT mice, no tumor vaccination could be achieved with anthracycline- or oxaliplatin-treated cells in Tlr4$^{-/-}$ mice (**Fig. 4a**). These data were confirmed for oxaliplatin-treated or irradiated EL4 thymoma cells, which failed to protect Tlr4$^{-/-}$ hosts against tumor challenge (**Fig. 4a**). Pharmacological inhibition of TLR4 with a cell-permeable blocking peptide[16] that was co-injected with dying tumor cells also prevented antitumor immunity (**Fig. 4b**). Moreover, the depletion of HMGB1 from doxorubicin- or oxaliplatin-treated tumor cells using neutralizing antibodies (**Fig. 4c**) or HMGB1-specific siRNAs (**Fig. 4d**) compromised the efficacy of antitumor vaccination.
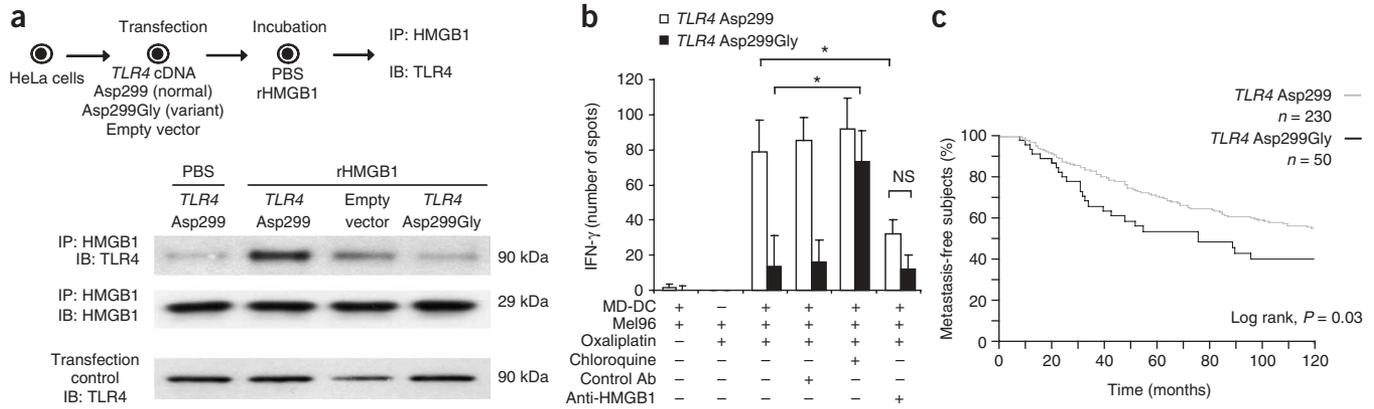
Figure 6 TLR4 dictates the efficacy of antitumor chemotherapy in humans. (**a**) Defective binding of HMGB1 to TLR4 conferred by the Tlr4 Asp299Gly polymorphism. HeLa cells were transfected with a vector containing the Tlr4 Asp299 (normal) cDNA or the Tlr4 Asp299Gly mutated cDNA or an empty vector. Transfectants were incubated in the presence of rHMGB1 for 1 h and immunoprecipitation assays were done as indicated. (**b**) Defective capacity of human DCs harboring the TLR4 Asp299Gly mutation to cross-present tumor antigens to CTL clones. HLA-A2–positive MD-DCs were cocultured with dying HLA-A2–negative melanoma cells expressing Mart1 as well as a CTL clone specific for A2/Mart1. IFN-γ ELISPOT assays were conducted to assess CTL activation. The graph represents the mean ± s.e.m. of the number of spots in each condition in a representative experiment (out of two) in a single donor for each genotype. The results obtained with a second pair of individuals are depicted in **Supplementary Figure 5**. (**c**) Kaplan-Meier estimates of time to metastasis between two groups of patients bearing the normal or mutated Tlr4 alleles. The time to progression was analyzed in 280 women with non-metastatic breast cancer with lymph node involvement who were treated by surgery followed by anthracycline-based chemotherapy and local irradiation.

In the treatment of established tumors with systemic chemotherapy or local radiotherapy, the presence of TLR4 dictated the therapeutic outcome. CT26 colon cancers (**Fig. 5a**), TS/A breast carcinomas (**Fig. 5b**), heterotransplanted GOS osteosarcomas (**Fig. 5c**) and EL4 thymomas (**Fig. 5d**) progressed with similar kinetics in immuno-competent WT, Tlr4^{−/−} and nu/nu athymic mice. Chemotherapy with appropriate cytotoxic agents or local radiotherapy reduced tumor growth and prolonged the survival of tumor-bearing mice in immuno-competent WT mice yet was less effective in Tlr4^{−/−} (**Fig. 5a–d**) and nu/nu mice (**Fig. 5b** and data not shown). In accordance with the in vitro data (**Fig. 1a**), Trif^{−/−} mice mounted a similar chemo-therapeutic response as WT mice, whereas Myd88^{−/−} mice behaved like Tlr4^{−/−} mice (**Fig. 5c**). Moreover, systemic administration of chloroquine enhanced the efficacy of chemotherapy in Tlr4^{−/−} mice but not in WT mice (**Fig. 5d**), in agreement with data presented in **Supplementary Figure 3**. These results point to a hitherto unrecognized contribution of TLR4/MyD88-dependent immunity to chemotherapeutic regimens.

Relevance of a TLR4 mutant for chemotherapy efficacy

A sequence polymorphism in Tlr4 (896A/G, Asp299Gly, rs4986790) affecting the extracellular domain of TLR4 is associated with reduced endotoxin responses and with a reduced susceptibility to cardiovascular disease in humans[28,29]. Although it is a matter of debate whether the TLR4 Asp299Gly mutation results in deficient LPS signaling[28,30,31], we addressed the possibility that the TLR4 Asp299Gly polymorphism could affect the response to HMGB1. Immunoprecipitation experiments were performed after adding HMGB1 to HeLa cells that were transfected with normal and mutated human TLR4. The binding of HMGB1 to the mutant (Asp299Gly) TLR4 was reduced as compared to its binding to the normal (Asp299) TLR4 (**Fig. 6a**), although both transfectants expressed similar numbers of TLR4 molecules. This defective binding of HMGB1 to the mutated TLR4 might account for the severely impaired capacity of monocyte-derived DCs (MD-DCs) to cross-present melanoma antigens to CTLs. MD-DCs from normal (Asp299) individuals cross-presented Mart1 derived from dying melanoma cells to CTL clones in an HMGB1-dependent

manner (**Fig. 6b** and **Supplementary Fig. 5** online). In contrast, MD-DCs from individuals bearing an Asp299Gly TLR4 allele did not cross-present (**Fig. 6b** and **Supplementary Fig. 5**). This defect was restored by addition of chloroquine (**Fig. 6b** and **Supplementary Fig. 5**). Thus, TLR4 Asp299Gly inhibits the HMGB1 response.

The breast cancer patients who benefit the most from adjuvant systemic administration of anthracyclines are those presenting with lymph node involvement. Therefore, we analyzed the time to metastasis in a cohort of 280 patients with non-metastatic breast cancer who were treated with anthracyclines after local surgery revealing lymph node involvement. The frequencies of heterozygous and homozygous germline polymorphisms encoding Asp299Gly were 17.1% and 0.7%, respectively (the two will be referred to collectively as 'mutated TLR4' hereafter). Patients carrying the mutated TLR4 allele did not differ from patients with the normal TLR4 allele with regard to any classical prognostic factors (see **Supplementary Table 1** online). The frequency of metastasis by 5 years after surgery was statistically higher in the group carrying a mutated TLR4 (40%, versus 26.5% in patients without the mutation; $P < 0.05$, relative risk 1.53, 95% confidence interval 1.1–3.58). Moreover, the Kaplan-Meier estimate of metastasis-free survival showed an overall significantly lower percentage of metastasis-free patients in the group with mutated TLR4 (log-rank test, $P = 0.03$) (**Fig. 6c**). In contrast, single-nucleotide polymorphisms affecting the TLR4 intron or 5′ untranslated region (TLR4 mutations rs1927911 and rs10759932 (ref. 32), respectively) had no correlation with the metastasis-free survival of the same cohort of patients (data not shown).

Hence, a specific mutation of TLR4 with functional relevance may influence the immunological component of anthracycline-based chemotherapy in human cancer.

DISCUSSION

Cancer patients and their physicians who receive and apply chemotherapy, respectively, do so in the genuine belief that the prime goal of therapy is to destroy tumor cells. Here, we show for the first time that anticancer chemotherapy has an additional, decisive effect. Dying tumor cells elicit 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 patients. We have defined (one of) the molecular mechanism(s) that dictates the chemotherapy-elicited antitumor immune response, namely the functional interaction between one compound released from dying tumor cells (HMGB1) and one particular receptor that is important for the function of the immune system (TLR4).

Injured tissue can trigger acute and transient immune responses against self antigens[1,33], presumably because dying cells release adjuvant factors that amplify and sustain DC- and T cell–dependent immune responses[34–38]. Recent studies have described the roles of IFN type 1 and the *N*-ethyl-*N*-nitrosourea–induced germline mutation 3d in the T cell–dependent immunogenicity of Fas- and UV-induced apoptotic splenic cells expressing a membrane-associated form of ovalbumin[35,36]. Several damage-associated molecular patterns, including hyaluronans, HSPs and fibronectin, have been identified as TLR4 ligands[39]. However, endogenous 'danger' signals have thus far not been implicated in antitumor immune responses. Here, we show that dying tumor cells produced by cancer therapies trigger a cognate immune response in a TLR4-dependent fashion (**Figs. 1** and **2**). TLR4 has previously been reported to play a part in lung tumorigenesis induced through chemically induced pulmonary inflammation[40]. Nonetheless, this observation did not link TLR4 expression to the induction of specific antitumor immune responses.

In the present study, we were able to identify one particular TLR4 ligand, HMGB1, as indispensable for the death-driven immunoadjuvant effects of chemotherapy. HMGB1 is a nonhistone chromatin-binding nuclear constituent that is passively released by dying cells and actively secreted by inflammatory APCs[25,41]. HMGB1 is a mediator of inflammation in the extracellular environment that exerts an important pathogenic role in late sepsis[26] as well as in hepatic ischemia-reperfusion; TLR4 is known to have a role in the latter process[24]. HMGB1 released by dead cells is a potent adjuvant *in vivo*[27]. Moreover, HMGB1 binding to its receptor, the receptor for advanced glycation end products (RAGE), promoted DC activation and elicited immune responses[42]. A recent report underscored the capacity of HMGB1 to trigger DC migration[43]. Here we provide evidence of a physical and functional interaction between HMGB1 released by tumor cells and TLR4 (**Figs. 3**, **4** and **5**). We were able to exclude the possibility that HMGB1 is required for the maturation of mouse and human DCs (**Supplementary Fig. 2b,c** and data not shown). Rather, our results suggest that TLR4 prevents the accelerated degradation of the phagocytic cargo within DCs, thereby allowing for optimal antigen presentation (**Supplementary Fig. 3**). Cross-presentation of tumor antigens derived from dying tumor cells by mouse or human DCs to T-cell hybridoma or CTL clones was selectively impaired in TLR4-deficient DCs and was HMGB1 dependent (**Supplementary Figs. 4** and **5** and **Fig. 6b**). The exact mechanism by which HMGB1-TLR4 interactions influence the processing and presentation of tumor antigens has yet to be deciphered.

Our data suggest 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. This polymorphism can affect the response of epithelial bronchial cells and alveolar macrophages to inhaled LPS[28]. Our experiments revealed that the *TLR4* Asp299Gly single-nucleotide polymorphism (SNP) reduces the interaction between TLR4 and HMGB1 (**Fig. 6a**) and abolishes the capacity of MD-DCs to cross-present dying melanoma cells to Mart1-specific HLA-A2–restricted-CTLs, a biological property that depends on

HMGB1 in WT MD-DCs (**Fig. 6b**). However, the altered cross-presentation ability conferred by the *TLR4* Asp299Gly SNP was restored by culturing MD-DCs with chloroquine (**Fig. 6b**), a treatment that also overcame the defect of antigen presentation by *TLR4*−/− mouse DCs *in vitro* (**Supplementary Fig. 3a,b**) and *in vivo* (**Fig. 5d**).

Notably, 17% of the group of breast cancer patients presenting with lymph node involvement carried the *TLR4* Asp299Gly allelic variant, and these *TLR4* Asp299Gly carriers showed a shorter time to progression (**Fig. 6c**). To the best of our knowledge, this is the first report revealing that immunogenetic factors might affect clinical outcome in breast cancer.

Altogether, the immunoadjuvant effect of radiotherapy and chemotherapy relies upon two major checkpoints, calreticulin exposure (the 'eat me' signal) and HMGB1 release (the 'danger' signal) by dying tumor cells, thus licensing DCs for antigen uptake[8] and TLR4-dependent antigen processing, respectively. Only when both the 'eat me' and the 'danger' signals are correctly emitted by dying tumor cells and perceived by DCs will an immune response ensue. This knowledge may be clinically exploited to enhance the immunogenicity of current chemotherapeutic regimens. A major challenge will be to determine whether the immune defect induced by deficient TLR4 signaling can be alleviated by combining chemotherapy with alternate TLR agonists or with lysosomal inhibitors such as chloroquine.

METHODS

Mouse strains. All animals were bred and maintained according to both the FELASA and the Animal Experimental Ethics Committee Guidelines (Val de Marne, France). Animals were used at between 6 and 20 weeks of age. C57BL/6 *Tlr1*−/−, *Tlr2*−/−, *Tlr3*−/−, *Tlr4*−/− (ref. 44), *Tlr5*−/−, *Tlr6*−/−, *Tlr7*−/−, *Tlr9*−/−, *Trif*−/− and *Myd88*−/− mice were gifts (see Acknowledgments). Genetic background and origin of other mice are detailed in the **Supplementary Methods** online.

Tumor cell lines and transplantable tumors. CT26 colon cancer cells (syngenic from BALB/c mice), TS/A breast cancer cells (syngenic from BALB/c mice), TS/A-OVA breast cancer cells (syngenic from BALB/c mice), EL4 thymoma cells (syngenic from C57BL/6 mice), EG7 cells (OVA-transfected EL4 cells) and MCA205 fibrosarcoma cells (syngenic from C57BL/6 mice) were cultured at 37 °C under 5% CO_2 in endotoxin-free RPMI 1640 medium supplemented with 10% FCS, penicillin and streptomycin[6], 1 mM pyruvate and 10 mM HEPES. The Glasgow osteosarcoma (GOS) tumor was maintained in C57BL/6 mice over 6 weeks of age and transplanted every 2 weeks as s.c. implants[45].

Bone marrow–derived DCs and T-cell hybridoma assays. We propagated bone marrow–derived DCs as already described[46]. Further information is provided in the **Supplementary Methods**.

Immunoblot analysis. For immunoblot analysis, cells were lysed in lysis buffer. Whole-cell lysates, purified plasma membranes or supernatants were resolved by SDS-PAGE and then transferred onto nitrocellulose membrane and probed with appropriate primary and secondary antibodies. Further details are described in the **Supplementary Methods**.

RNA interference knockdown of HMGB1. We transfected CT26, MCA205 and EG7 cells using HiPerFect (CT26, MCA205) (Qiagen) or nucleofection using Nucleofector Kit L (EG7) (Amaxa) with either PBS, irrelevant siRNA, HMGB1 siRNA 1 or HMGB1 siRNA 2 (siRNA sequences are detailed in the **Supplementary Methods**).

Detection of peptide–MHC class I complexes at the surface of DCs using specific antibody to 25D1.16 (ref. 47). We performed detection of peptide–MHC class I complexes at the surface of DCs as previously described. Further details are provided in the **Supplementary Methods**.

Anticancer vaccination. CT26, EL4 and MCA205 cells were cultured with either PBS, doxorubicin (20 μM for CT26 and 1 μM for MCA205) or

oxaliplatin (5 μg/ml) (Sanofi-Aventis) for 24 h. Alternatively, EL4 cells were subjected to 10 Gy of X-ray irradiation (RT250, Phillips). In some experiments cells were transfected with HMGB1 siRNA or irrelevant siRNA 48 h before *in vitro* treatment. All these treatments resulted in a population containing ∼30% annexin V$^+$ DAPI$^+$ double-positive cells, as assessed by FACS analysis at 24 h. 3 × 10^6 dying CT26 cells or 5 × 10^6 dying EL4 cells were injected s.c. into the left flanks of mice. Seven days later, mice were re-challenged in the right flank with 5 × 10^5 live CT26 or EL4 cells. Tumor growth was then monitored weekly using calipers.

Chemotherapy and radiotherapy of established tumors in mice. WT or loss-of-function mice were injected in the flank with 10^5 EL4 CT26 TS/A cells. Mice were then randomly assigned into treatment groups of 4–6 mice each. Tumor surface was monitored using calipers. When tumor size reached 40–80 mm^2, mice were treated with oxaliplatin (5 mg per kg body weight i.p for EL4), doxorubicin (2 μM injected intratumorally in 100 μl PBS, for CT26) or local X-ray irradiation (for TS/A). For local radiotherapy, mice were briefly anesthetized using isoflurane, placed into plastic constrainers and locally irradiated (10 Gy). The whole body was protected by lead shielding, except for the area of the tumor to be irradiated. For Glasgow osteosarcoma (GOS), oxaliplatin (5 mg/kg intraperitoneally (i.p.)) was administered at day 5 when tumors became palpable[45].

Genotyping of TLR4 Asp299Gly, Thr399Ile and related SNPs. DNA was isolated from frozen blood leukocytes from subjects. PCR primers (Applied Biosystems) were used to amplify a 101-bp fragment containing the *TLR4* Asp299Gly mutation (rs4986790) site. After PCR amplification, genotypes were assigned to each subject, by comparing the signals from the two fluorescent probes, FAM and VIC, and calculating the –log(FAM/VIC) ratio for each data point[48]. Other *TLR4* polymorphisms and CD14-260 C/T SNPs were also analyzed in parallel using predesigned PCR primers from Applied Biosystems.

Statistical analyses. For the analysis of experimental data, comparison of continuous data was achieved by the Mann-Whitney U test and comparison of categorical data by χ^2 or Fisher's exact test, as appropriate. The log-rank test was used for analysis of Kaplan-Meier survival curves. All statistical analyses were performed with JMP 5.1 (SAS Institute). All P values are two tailed. A P value <0.05 was considered statistically significant for all experiments.

Additional information about reagents and materials, anticancer vaccination, priming experiments, immunoprecipitation and clinical study design are detailed in the **Supplementary Methods**.

Note: Supplementary information is available on the Nature Medicine website.

AUTHOR CONTRIBUTIONS
L.A., F.G., A.T., M.O., G.M., M.C.M. and E.U. performed the *in vivo* and *in vitro* experiments. C.O. performed *in vitro* experiments. A.C. performed immunoprecipitations. B.R. provided transgenic mice. F.J.B., H.Y. and F.L. provided essential reagents. R.L., C.N., J.-P.M., A.C., V.J., F.C.-C., S.D. and T.T. recorded and provided the patients' data. L.A. and P. Saulnier performed patients' genotyping. A.T., F.A. and F.G. conducted data analysis. S.A. offered scientific

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