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ORIGINAL ARTICLE

Immunogenic death of colon cancer cells treated with oxaliplatin

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Both the pre-apoptotic exposure of calreticulin (CRT) and the post-apoptotic release of high-mobility group box 1 protein (HMGB1) are required for immunogenic cell death elicited by anthracyclins. Here, we show that both oxaliplatin (OXP) and cisplatin (CDDP) were equally efficient in triggering HMGB1 release. However, OXP, but not CDDP, stimulates pre-apoptotic CRT exposure in a series of murine and human colon cancer cell lines. Subcutaneous injection of OXP-treated colorectal cancer (CRC), CT26, cells induced an anticancer immune response that was reduced by short interfering RNA-mediated depletion of CRT or HMGB1. In contrast, CDDP-treated CT26 cells failed to induce anticancer immunity, unless recombinant CRT protein was absorbed into the cells. CT26 tumors implanted in immunocompetent mice responded to OXP treatment *in vivo*, and this therapeutic response was lost when CRT exposure by CT26 cells was inhibited or when CT26 cells were implanted in immunodeficient mice. The knockout of toll-like receptor 4 (TLR4), the receptor for HMGB1, also resulted in a deficient immune response against OXP-treated CT26 cells. In patients with advanced (stage IV, Duke D) CRC, who received an OXP-based chemotherapeutic regimen, the loss-of-function allele of TLR4 (Asp299Gly in linkage disequilibrium with Thr399Ile, reducing its affinity for HMGB1) was as prevalent as in the general population. However, patients carrying the TLR4 loss-of-function allele exhibited reduced progression-free and overall survival, as compared with patients carrying the normal TLR4 allele. In conclusion, OXP induces immunogenic death of CRC cells, and this effect determines its therapeutic efficacy in CRC patients.
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Introduction

Conventional anticancer chemotherapy or radiotherapy is generally thought to reduce tumor progression by direct cytostatic and cytotoxic effects on tumor cells. Although this paradigm undoubtedly holds for human cancers that are xenografted into immunodeficient mice, it must be revised for tumors that grow on immunocompetent hosts (Apetoh *et al*., 2007a, b). Thus, multiple different murine cancer models, including CT26 colon cancers, EL4 thymomas, TS/A mammary carcinomas, MCA205 fibrosarcomas and GOS osteosarcomas respond more efficiently to conventional anticancer therapy when they are implanted in syngeneic immunocompetent as opposed to immunodeficient hosts (Apetoh *et al*., 2007a, b). On the basis of these observations, we and other researchers have become interested in the contribution of the immune system to the efficacy of anticancer chemotherapies.

Many chemotherapeutic agents mediate their cytotoxic effects by the induction of apoptosis, and apoptosis is generally thought to be non-inflammatory and non-immunogenic (Savill and Fadok, 2000; Matzinger, 2002). Recent data, however, suggest that, in spite of its morphological uniformity, apoptosis can follow biochemically distinct subroutines, some of which may result in immunogenic cell death (Blachere *et al*., 2005; Casares *et al*., 2005; Sancho *et al*., 2008; Laane *et al*., 2009). When murine tumor cells are treated with distinct apoptosis inducers and then injected subcutaneously, in the absence of an adjuvant, they mostly fail to elicit an immune response that would protect the animals against subsequent challenge with live tumor cells (Casares *et al*., 2005; Obeid *et al*., 2007b). In contrast to many other apoptosis-inducing regimens, anthracyclines can induce immunogenic cell death, meaning that tumor cells that die in response to anthracyclines constitute efficient anticancer vaccines and elicit the generation of

tumor-specific cytotoxic T lymphocytes, in a dendritic cell (DC)-dependent manner (Apetoh *et al*., 2007b; Tesniere *et al*., 2008; Sancho *et al*., 2009).

In contrast to cell death elicited by standard protocols of apoptosis induction (such as treatment with the general tyrosine kinase inhibitor, staurosporin, or the topoisomerase inhibitors etoposide and campothecin), anthracyclin-induced cell death is preceded by the pre-apoptotic exposure of calreticulin (CRT) on the plasma membrane surface (Obeid *et al*., 2007b). Although CRT is usually located in the lumen of the endoplasmic reticulum (ER), yet it translocates to the cell surface in response to anthracyclines. This involves a complex signal transduction pathway, including an ER stress response, the subapoptotic activation of caspase-8 and the exocytosis-dependent co-translocation of CRT, together with another ER protein, ERp57, to the outer surface of the plasma membrane (Laane *et al*., 2009). Surface CRT serves as an engulfment signal (Obeid *et al*., 2007a, b) and similarly targets apoptotic cells for interaction with DCs and subsequent cross-presentation of tumor antigens (Zeng *et al*., 2006). Inhibition of CRT exposure by knockdown of CRT or ERp57 abolishes the immunogenicity of cell death (Obeid *et al*., 2007b; Panaretakis *et al*., 2008), and ERp57-deficient cells implanted into mice form anthracyclin-resistant tumors (Panaretakis *et al*., 2008). Importantly, non-immunogenic cell death (without CRT exposure) can be rendered immunogenic by absorbing recombinant CRT protein to the cell surface (Obeid *et al*., 2007a, b), and chemotherapeutic agents that fail to induce CRT exposure can be rendered efficient by injecting CRT into tumors (Obeid *et al*., 2007b). Local CRT injection also reverses the chemotherapy resistance of ERp57-deficient tumors (Panaretakis *et al*., 2008). These results establish CRT exposure as a major checkpoint of immunogenic cell death.

To be detected as immunogenic, dying cells must emit signals in addition to CRT. Indeed, dying cells release HMGB1 into the extracellular milieu in response to most chemotherapeutic agents (Apetoh *et al*., 2007a, b), and neutralization or depletion of HMGB1 abolishes the immunogenicity of cell death (Apetoh *et al*., 2007b). HMGB1 can interact with several receptors expressed on the surface of DCs (Park *et al*., 2006) including toll-like receptor 4 (TLR4). Mice that lack TLR4 or that bear a mutant TLR4 fail to mount an immune response against anthracyclin-treated cancer cells (Apetoh *et al*., 2007b). Moreover, tumors growing on TLR4-deficient mice are largely refractory to anticancer chemotherapy (Apetoh *et al*., 2007b). This result can be recapitulated in humans. Approximately 12–14% of Caucasians bear an allele of TLR4 in which two residues (amino acids 299 and 399) of the extracellular loop are mutated (Ferwerda *et al*., 2007). This results in a mutated TLR4 protein (Asp299Gly, Thr399Ile) that exhibits a reduced affinity for HMGB1 (Apetoh *et al*., 2007b). Importantly, breast cancer patients bearing this loss-of-function allele of TLR4 exhibit an accelerated relapse after anthracyclin-based adjuvant chemotherapy compared with patients bearing the normal TRL4 allele (Apetoh *et al*., 2007b), underscoring the importance of anti-cancer immunity for an optimal response to anthracyclines (Zitvogel *et al*., 2008).

Circumstantial evidence suggests that, similar to anthracyclines, oxaliplatin (OXP) can induce immunogenic cell death. Thus, ovalbumin-expressing thymoma cells (EG7 cells) were found to efficiently prime popliteal T cells *in vivo* when they were treated with OXP and injected into the footpad of immunocompetent mice (Apetoh *et al*., 2007b). This T-cell priming was suppressed by the neutralization of HMGB1 and required the expression of TLR4 by DCs (Apetoh *et al*., 2007b). Oxaliplatin is mostly used for the treatment of CRC (Muggia and Fojo, 2004; Kelland, 2007), a tumor type prognosis of which is profoundly influenced by the type, density and location of lymphoid infiltrates in tumor beds (Pages *et al*., 2005; Galon *et al*., 2006). Thus, even in advanced CRC, the presence of markers of cytotoxic T cells and T helper 1-adaptative responses has a positive prognostic impact (Galon *et al*., 2006; Camus *et al*., 2009).

Driven by these considerations, we decided to evaluate the possible role of OXP as an immunogenic cell death inducer in the treatment of CRC. In this study, we show that OXP (but not cisplatin, CDDP) induces immunogenic apoptosis accompanied by CRT exposure, that OXP-mediated chemotherapy of colon cancers implanted into mice relies on an intact immune system including the presence of TLR4, and that advanced CRC patients bearing the loss-of-function allele of TLR4 had a lower progression-free survival (PFS) after OXP-based chemotherapy compared with control patients with a wild-type allele.

Results

Oxaliplatin induces immunogenic colon cancer cell death

In response to CDDP or OXP, CT26 cells underwent cell death, as indicated by staining with annexin-V fluorescein isothiocyanate (which detects phosphatidylserine residues on the surface of apoptotic cells) and the vital dye, propidium iodine (that only penetrates into dead cells; Figures 1a and b). Kinetic and morphological analyses indicated that morphologically discernible apoptosis was followed by secondary necrosis (data not shown). As a positive control (Obeid *et al*., 2007b), mitoxantrone was used to induce immunogenic cell death. Thus, Balb/c mice inoculated with mitoxantrone-treated CT26 cells as a tumor vaccine, were protected against a subsequent challenge with live CT26 cells. Although CDDP-treated tumor cells largely failed to induce an immune response, OXP-treated CT26 cells vaccinated as efficiently against cancer as mitoxantrone-treated cells (Figures 1c and d), pointing to a major difference between OXP and CDDP, which induced immunogenic versus non-immunogenic cell death, respectively.

Next, we treated established CT26 colon cancers as soon as they became palpable with CDDP (50 μg/kg) or OXP (250 μg/kg). This treatment caused a delay in the growth of CT26 tumors implanted in immunocompetent

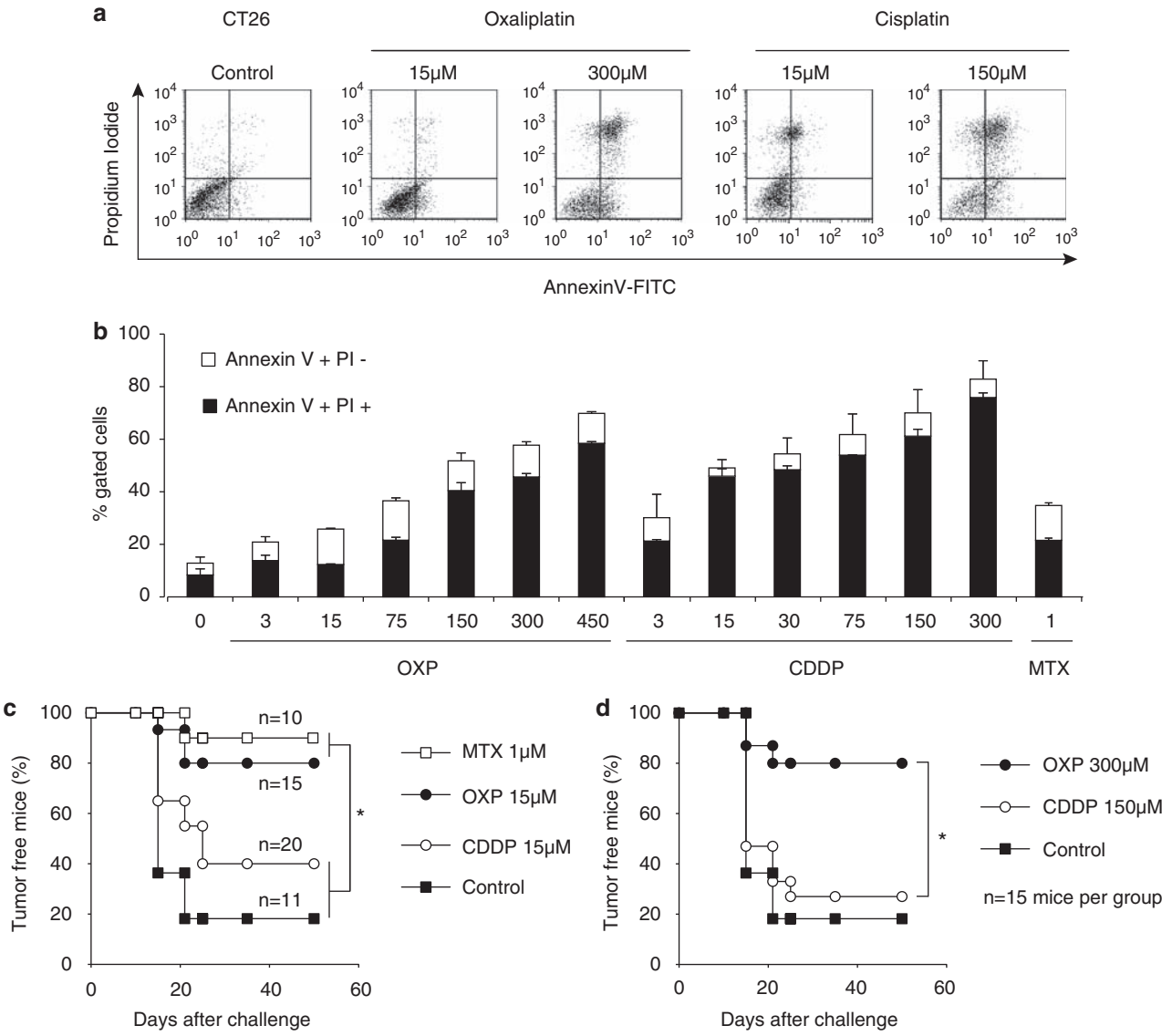

Figure 1 Oxaliplatin (OXP) induces immunogenic colon cancer cell death. (**a**, **b**) Frequency of apoptotic (Annexin V^+ PI^-) and secondary necrotic (Annexin V^+ PI^+) CT26 cells after 24 h of treatment with OXP, cisplatin (CDDP) and mitoxantrone (MTX). CT26 cells were cultured in complete medium along with chemotherapeutic agents, at the indicated dose, for 24 h, and then stained with annexin-V fluorescein isothiocyanate (FITC) and with vital dye, propidium iodine, before subsequent analysis by flow cytometry. Data are represented as dot plot (**a**) or mean value ± s.e.m. of triplicates of one representative experiment (**b**). (**c**, **d**) OXP-treated CT26 cells vaccinated efficiently against live tumor cells. CT26 cells treated *in vitro* with OXP, CDDP or MTX were inoculated subcutaneously in Balb/c mice. After 7–10 days, mice were re-challenged with live 5×10^6 CT26 cells. The percentages of tumor-free mice are pooled from three independent experiments. $^*P<0.05$.

Balb/c mice, with OXP yielding a more pronounced therapeutic response than CDDP (Figure 2a). A dose escalation using OXP or CDDP by a factor of 10 did not improve the therapeutic outcome (Supplementary Figure 1). Importantly, no therapeutic effect was observed when instead of CT26 wild-type cells, CT26 cells, subjected to a stable knockdown of ERp57 using a short hairpin RNA, were injected into immunocompetent Balb/c mice and then treated with CDDP or OXP (Figure 2b). Such ERp57 knockdown cells failed to emit immunogenic signals (such as CRT exposure) (Panaretakis *et al*., 2008) and hence become refractory to chemotherapies whose efficiency relies on the immune system (Tesniere *et al*., 2008). To further investigate the contribution of the immune system to the control of tumor growth, we treated CT26 tumors that were expanding in immunodeficient *nu*/*nu* mice with CDDP or OXP. Neither of the two treatments retarded the growth of tumors implanted in *nu*/*nu* mice (Figure 2c), underscoring the contribution of the immune system to the therapeutic response elicited by OXP (and to less extent by CDDP).

Calreticulin exposure determines the immunogenicity of OXP-induced cell death

After short-term stimulation (4 h) with mitoxantrone or OXP, CT26 cells exposed CRT on the cell surface,

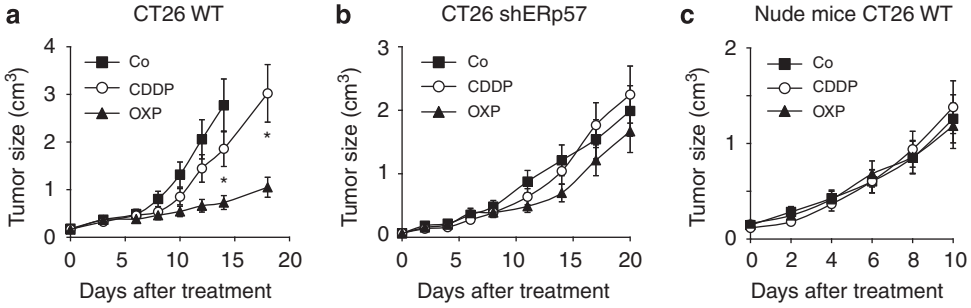

Figure 2 Calreticulin (CRT) exposure and the immune system are critical for the therapeutic effect of oxaliplatin (OXP) on established tumors *in vivo*. CT26 colon cancer tumors were subcutaneously established in immunocompetent (wild—type mice) (**a**, **b**) or immunodeficient (*nu/nu)* mice (**c**). **(b)** CT26 cells were previously stably transfected with short hairpin RNA for ERp57, which diminish the ability of CRT exposure on the cell surface and then the efficacy of immunogenic chemotherapy. When the tumors reached 40–80 mm^2 in size, mice were either left untreated or treated with intratumoral chemotherapy (OXP: 1.25 mg/kg; CDDP: 0.25 mg/kg). Tumor sizes were monitored every 2 or 3 days using a caliper and tumor volumes were calculated as previously described (35). Each group included five mice. One representative experiment out of three is depicted. $*P<0.05$.

as determined by immunofluorescence staining and microscopic examination (Figure 3a) or cytofluorometric analysis (Figure 3b). In identical conditions, however, CDDP failed to elicit pre-apoptotic CRT exposure, over a wide range of different concentrations (Figures 3a-c). Depletion of CRT using an siRNA abolished the immunogenicity of OXP-treated CT26 cells *in vivo* (Figure 3d). Absorbance of recombinant CRT protein to the plasma membrane restored the lost immunogenicity of CRT-depleted OXP-treated cells (Figure 3d), as well as the absent immunogenicity of CDDP-treated CT26 cells (Figure 3e). Similarly, CDDP-treated EG7 cells (which express the model antigen OVA) were unable to prime popliteal T cells for interferon-γ production *in vivo*, and this defective T-cell priming was restored by absorbing recombinant CRT protein onto the surface of cells before they were injected into the footpad (Figure 3f). These results point to a fundamental difference in the ability of the two platinum compounds in eliciting CRT exposure, which is necessary for signaling immunogenicity. The difference between CDDP and OXP with regard to their CRT-exposing activity was observed over a range of different colon cancer cell lines. Thus, the two human colon cancer cell lines, RKO and HCT116, both of which were killed by CDDP and OXP (Supplementary Figure 2), exhibited CRT exposure in response to OXP, but not in response to CDDP (Supplementary Figure 3). Altogether, these results support the idea that OXP is a more efficient inducer of CRT exposure than CDDP, and that this difference in CRT exposure determines the divergence in the *in vivo* anti-tumor efficacy of both drugs.

The HMGB1–TLR4 axis contributes to the immunogenicity of OXP-induced cell death

Both OXP and CDDP induced the release of HMGB1 from the nuclei of mouse colon cancer cells (Figure 4a). HMGB1 appeared in the supernatant of the cells, as detectable by enzyme-linked immunosorbent assay (Figure 4b). Similarly, both OXP and CDDP released HMGB1 from several human colon cancer cell lines (Supplementary Figure 4). Neutralization of HMGB1 with a specific antibody abolished the capacity of OXP-treated EG7 cells to prime T cells for interferon-γ production (Figure 4c). Similarly, the co-injection of the neutralizing anti-HMGB1 antibody abolished the capacity of OXP-treated tumor cells to vaccinate against CT26 cancers. The partial depletion of HMGB1 with a specific siRNA also reduced the immunogenicity of OXP-treated tumor cells (Figure 4d). Moreover, OXP-treated dying cells failed to elicit an anti-tumor immune response in $tlr4^{-/-}$ mice, in conditions in which $tlr4^{+/+}$ controls were protected against challenge with live cancer cells (Figure 4e). These results corroborate the importance of the HMGB1–TLR4 axis for the immune response against tumor cells dying in response to OXP.

The Asp299Gly polymorphism influences PFS in advanced patients treated with chemotherapy

Next, we investigated whether a loss-of-function allele of *TLR4* (Asp299Gly, in linkage disequilibrium with Thr399Ile) (Ferwerda *et al*., 2007) could affect PFS of metastatic CRC patients (Bouché *et al*., 2007) ($n=338$) that had been included in the FFCD 2000–05 randomized trial and for whom blood samples were available. This trial compared the single agent 5-fluorouracil (administered together with folinic acid) until failure, then combination chemotherapy with OXP plus 5-fluorouracil versus the latter combination chemotherapy from the outset. The loss-of-function allele was approximately as frequent among the patient population (16.5%) as in the general Caucasian population (12–14%) (Arbour *et al*., 2000; Agnese *et al*., 2002). Patients that were heterozygous or homozygous for the *TLR4* Asp299Gly/Thr399Ile allele ($n=48$) were similar to patients bearing the normal *TLR4* allele ($n=290$) with respect to age, sex, number of disease sites, the WHO classification, alkaline phosphatase serum levels and other laboratory parameters (Table 1). Interestingly, patients bearing the normal *TLR4* allele manifested an increased progression-free survival

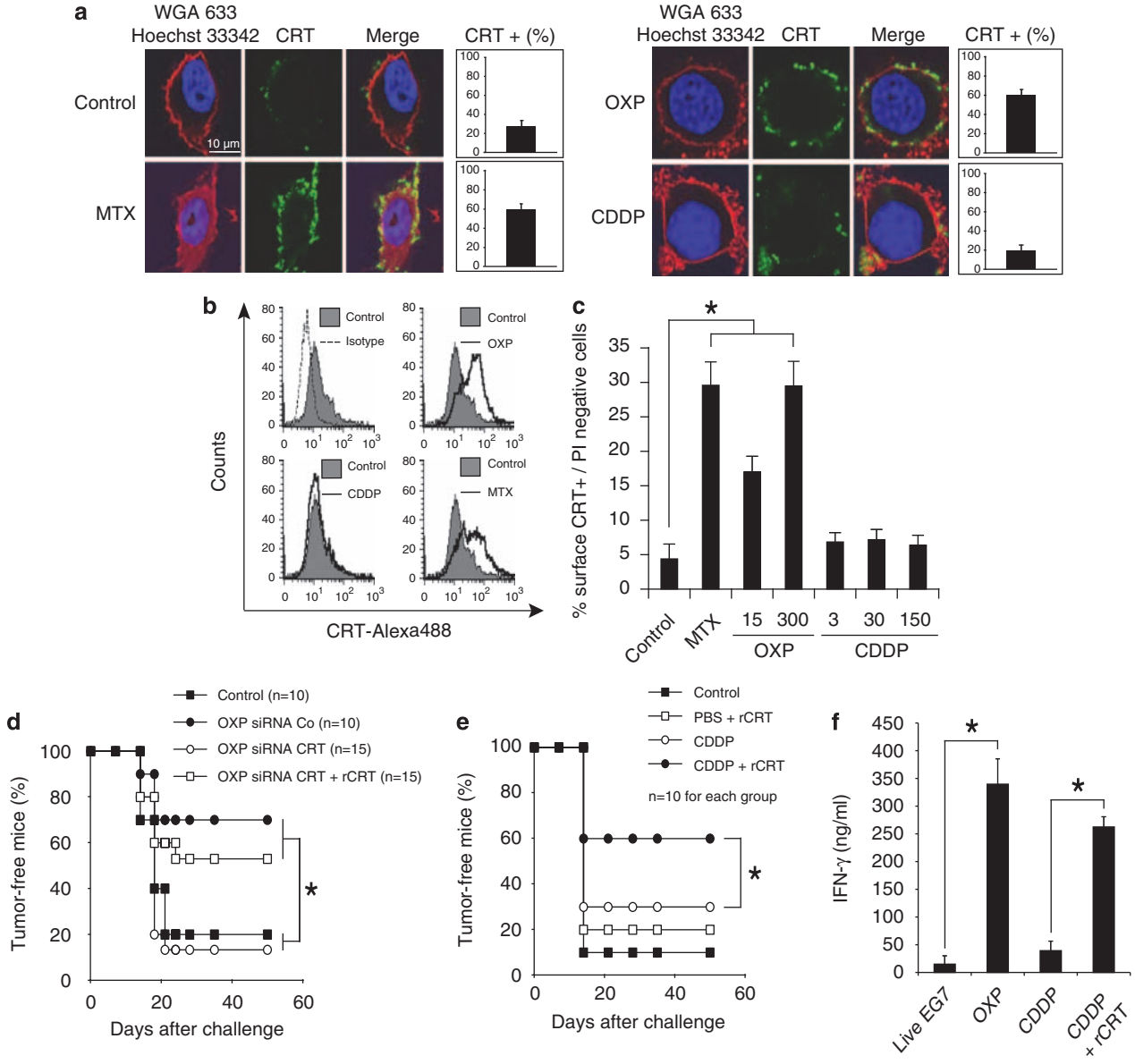

Figure 3 Calreticulin (CRT) exposure determines the immunogenicity of OXP-induced cell death. CRT exposure on the cell surface of CT26 cells was assessed after short-term stimulation (4 h) with mitoxantrone (MTX), oxaliplatin (OXP) or cisplatin (CDDP) treatment, or without treatment (control) by immunofluorescence staining followed by confocal microscopic examination (**a**) or by flow cytometric analysis (**b**, **c**). Data are presented as means ± s.e.m. of three independent experiments (A, B, C). (**d**, **e**) CRT exposure determines the efficacy of *in vivo* anti-cancer vaccination. (**d**) CT26 colon cancer cells were transfected with short interfering RNA(siRNA) for CRT (siRNA CRT) or an irrelevant siRNA (siRNA Co), and then treated with OXP for 24 h *in vitro*. In one group, recombinant CRT (rCRT) was coated on cells before the subcutaneous injection of the dying cells in one flank. At day 7, mice (*n* per group, as indicated) were inoculated with live syngeneic tumor cells in the opposite flank and tumor growth was monitored. The percentage of tumor-free mice is indicated. (**f**) CRT exposure determines the priming of T cells in the draining lymph node. OVA-expressing EG7 tumor cells were treated with medium alone, OXP (5 μM) or CDDP (5 μM) for 16 h and injected into the footpad of C57BL/6 mice. In some experiments, tumor cells treated with phosphate buffered saline (PBS) or CDDP were incubated with rCRT before *in vivo* injection. After 5 days, lymphocytes from the draining popliteal lymph nodes were challenged with PBS or PBS + OVA protein, and IFN-γ secretion was assessed at 72 h by ELISA. Data are presented as mean ± s.e.m. of IFN-γ secretion after re-stimulation with PBS–OVA or PBS alone. A representative out of three independent experiments is depicted. $^*P<0.05$.

(hazard ratio: 0.73; confidence interval = 0.53–1.00; $P \leqslant 0.05$) and overall survival (hazard ratio = 0.72; confidence interval = 0.52–1.01); $P = 0.05$), compared with patients bearing the loss-of-function allele of *TLR4* (Figure 5). We also investigated the role of the *tlr4*Asp299Gly polymorphism in a cohort of stage II CRC patients ($n = 258$) that were treated by surgical removal of the primary tumor in a curative intent, without any adjuvant chemotherapy. Furthermore, no differences were found in main patient and tumor characteristics according to *TLR4* genotype (data not shown). Interestingly, we found no statistical differences

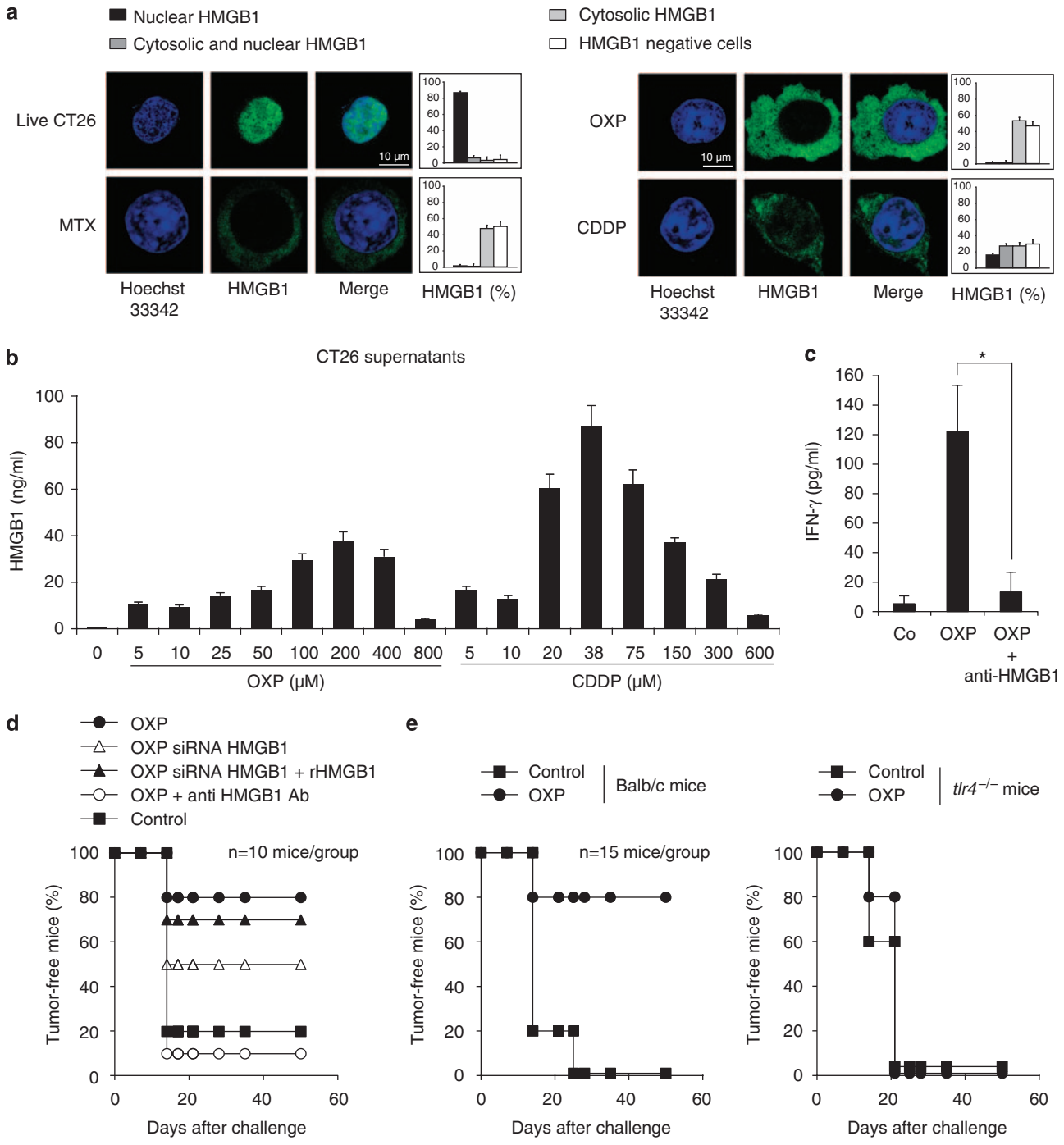

Figure 4 The HMGB1–TLR4 (high-mobility group box 1–toll-like receptor 4) axis contributes to the immunogenicity of oxaliplatin (OXP)-induced cell death. (**a**) Immunofluorescence microscopy of HMGB1 release from the nuclei to the cytosol in CT26 cells, untreated (live) or treated for 24 h with OXP (300 μM), cisplatin (CDDP, 150 μM) or mitoxantrone (MTX, 1 μM). After the indicated treatments, cells were permeabilized and stained with a rabbit anti-HMGB1 antibody (or an isotype-matched control antibody, data not shown) and were counterstained with a goat anti-rabbit Alexa 488. Nuclei were stained with Hoechst 33342. Data are presented as means ± s.e.m. of three independent experiments. (**b**) ELISA detection of HMGB1 release in the supernatants of CT26 cells treated with different concentrations of OXP and CDDP for 24 h. Data are presented as means ± s.e.m. of duplicates of a representative experiment. (**c**–**e**) HMGB1 determines the immunogenicity of dying tumor cells. The depletion of HMGB1 with a specific blocking antibody abolishes the priming of T cell responses to OXP-treated EG7 cells (see Figure 3f for details) (**c**) and the capacity of OXP-treated tumor cells to vaccinate against CT26 tumor cells (**d**). $*P<0.05$. The knockdown of HMGB1 in CT26 cells transfected with a specific small interfering RNA reduces the efficacy of antitumor vaccination, which can be restored by addition of recombinant HMGB1. Loss of the efficacy of antitumor vaccination with OXP-treated CT26 in *tlr4*$^{-/-}$ mice, in condition in which *tlr4*$^{+/+}$ controls were protected against challenge with live tumor cells.

Table 1 Baseline characteristics of patients and prognostic factors

	TLR4 Asp299Gly		*TLR4 Asp299Asp*		P-*value*
	n	%	*n*	%	
Sex					
Male	34	71	175	60	0.17
Female	14	29	115	40	
Age					
⩽60 years	11	23	104	36	0.21
Between 60 and 70 years	16	33	84	29	
>70 years	21	44	102	35	
WHO Score					
0 or 1	39	81	248	86	0.44
2	9	19	42	14	
Alcaline phosphatase					
Missing	1	2	19	7	0.4
⩽300 UI/l	33	69	206	71	
>300 UI/l	14	29	65	22	
White blood counts					
Missing	1	2	11	4	0.85
⩽10 000 /mm^3	34	71	198	68	
>10 000 /mm^3	13	27	81	28	
Number of disease sites					
1	25	52	154	53	0.9
>1	23	48	136	47	
Prior adjuvant chemotherapy					
No	43	90	255	88	0.74
Yes	5	10	35	12	
Prognostic score					
Missing	8	3	1	2	0.94
1	45	16	9	19	
2	103	36	16	33	
3	134	46	22	46	
Treatment					
LV5FU2 first	144	46	22	50	0.62
FOLFOX6 first	146	54	26	50	

Abbreviations: TLR4, toll-like receptor 4; WHO, World Health Organization.

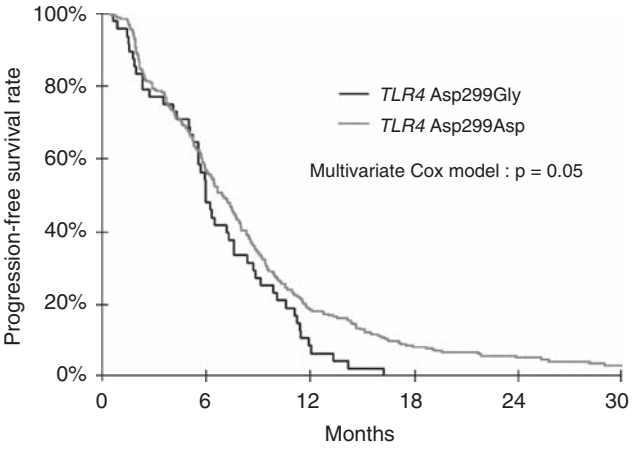

Figure 5 The Asp299Gly polymorphism influences progression-free survival in colorectal cancer patients treated with chemotherapy. Kaplan–Meier analysis of progression-free survival in metastatic colorectal cancer patients treated with oxaliplatin (OXP)-based chemotherapy regimen among patients bearing the *tlr4* Asp299Asp and Asp299Gly or Gly299Gly alleles.

in terms of disease-free survival among patients bearing the normal or variant allele of *TLR4*. This result shows that *TLR4*Asp299Gly is not a prognostic factor *per se,* unless patients receive immunogenic chemotherapy, thereby underlining the major role of immunogenic cell death induced by chemotherapy in the participation of the immune system for the therapeutic outcome. Altogether, these results indicate that the *TLR4* polymorphism Asp299Gly influences the therapeutic response of metastatic colon cancer patients toward OXP.

Discussion

Oxaliplatin and CDDP are chemically related agents that induce apoptosis through DNA damage as well as cytoplasmic effects (Wang and Lippard, 2005), and against which tumors often (but not always) exhibit cross-resistance (Castedo *et al*., 2006). In spite of their similarity, however, CDDP and OXP differ fundamentally in their capacity to elicit immunogenic cell death, as shown here. This difference was manifested at several levels, in different cell lines and species. Although OXP-treated cancer cells were able to prime T cells for interferon-γ production and to mediate anti-cancer vaccination, CDDP-treated cells failed to do so. At the biochemical level, the major difference between OXP- and CDDP-triggered cell deaths was found at the level of CRT exposure. OXP stimulated the translocation of CRT from the endoplasmic reticulum to the cell surface, whereas CDDP failed to do. Similarly, absent versus present CRT exposure explains the difference between CDDP versus OXP-induced cell death, respectively, because depletion of CRT rendered OXP-induced cell death non-immunogenic whereas absorption of CRT to CDDP-treated cells restored immunogenicity.

In contrast, OXP and CDDP were equipotent in inducing the release of HMGB1, which is another determinant of immunogenicity. It is important to note that failure to emit one single among several immunogenic signals (CRT exposure or HMGB1 release) is sufficient to abrogate the immunogenicity of cell death (Tesniere *et al*., 2008). Hence, the difference in CRT exposure is sufficient to explain the distinction between immunogenic versus non-immunogenic cell death caused by OXP and CDDP, respectively. This difference in the ability to trigger immunogenic cell death may be one plausible explanation to understand the lack of efficacy of CDDP in CRC patients.

It may be argued that only a minor fraction of OXP-treated tumor cells expose CRT at the pre-apoptotic stage (when the cells lack phosphatidylserine exposure and are viable), and that the amount of antigen that would be targeted to the DCs would be rather low. However, *in vivo*, when tumors are treated with

chemotherapy, dying cells were similarly phagocytosed before they lost the integrity of the plasma membrane and hence had a high chance of being engulfed by DCs. Indeed, as we inject oxaliplatin-treated tumor cells *in vivo*, a significant fraction of cells are still viable. As such oxaliplatin-treated CT26 cells do not form tumors *in vivo*, they must die after injection and similarly expose CRT before they lose viability. At this point, they might be engulfed by DCs that subsequently present tumor antigens to T lymphocytes.

The aforementioned results underscore the likely impact of the immune response on OXP-based chemotherapy. If an intact immune system is required for an optimal therapeutic response to OXP, two major predictions can be made. First, the chemotherapeutic response will not only be dictated by the capacity of tumor cells to undergo apoptosis in response to OXP but will also be influenced by their intrinsic potential to emit immunogenic signals (such as CRT exposure and HMGB1 secretion). In this context, it seems intriguing that reduced calreticulin expression constitutes a negative prognostic/predictive feature of colon cancers (Toquet *et al*., 2007). Reduced calreticulin expression also has a negative impact on neuroblastoma (Hsu *et al*., 2005), cervical carcinoma (Mehta *et al*., 2008), as well as on follicular thyroid carcinoma (Netea-Maier *et al*., 2008). Hence, it will be important to investigate whether CRT affects the immunogenicity of such tumors beyond its role in contributing to optimal peptide loading into major histocompatibility complex class I antigen (Wright *et al*., 2004; Zhang and Williams, 2006). Second, failure of the immune system to perceive immunogenic signals would have a negative influence on the efficacy of chemotherapy. In apparent accordance with this prediction, we found that the knockout of TLR4 compromised T-cell priming by OXP-treated EG7 thymoma cells (Apetoh *et al*., 2007b) and that TLR4 deficiency abolished the capacity of OXP-treated CT26 cells to induce a protective anti-cancer immune response (this study). TLR4 was also required for the optimal therapeutic response toward OXP in mouse models for thymoma and osteosarcoma (Apetoh *et al*., 2007a). Moreover, as shown here, a loss-of-function allele of *TLR4* was associated with reduced progression-free survival and overall survival in CRC patients receiving OXP-based chemotherapy for advanced disease.

In conclusion, OXP elicits immunogenic cell death in several rodent models of colon cancer. The immunogenicity of an OXP-induced cell death is governed by the same rules as applicable to those elicited by anthracyclines thus far that it involves CRT exposure and HMGB1 release. Moreover, it critically depends on the presence of functional TLR4 in the immune system. Correlative evidence suggests that these findings also apply to CRC patients in which a loss-of-function allele of *TLR4* negatively affects outcomes after OXP-based chemotherapy. On the basis of our results, we predict that *TLR4* alleles should not affect the therapeutic response to CDDP treatment, a problem that requires further investigation.

Materials and methods

Cell lines

CT26 and EG7 cells were cultured in RPMI 1640 medium, RKO and HCT116 cells in McCoys 5A (Gibco, Carlsbad, CA USA). All media were supplemented with 10% heat-inactivated fetal bovine serum, 10 mM Hepes, 10 U/ml penicillin and 10 μg/ml streptomycin.

Mouse strains

BALB/c (H-2d), C57BL/6 (H-2b) and *nu*/*nu* BALB/c mice were obtained from the Centre d'élevage Janvier (Le Genest St Isle, France) and from Charles River Laboratories (Saint-Germain sur l'Arbresle, France). BALB/c *Tlr4*$^{-/-}$ mice were kindly provided by Grégoire Lauvau (INSERM, University of Sofia Antipolis, Valbonne, France), and bred in our animal facility.

Antibodies and reagents

A rabbit polyclonal antibody against calreticulin (ab2907) and a rabbit polyclonal antibody against HMGB1 (ab18256) were purchased from Abcam (Paris, France). An Alexa 633-conjugated wheat germ agglutinin (WGA 633) was purchased from Invitrogen (Carlsbad, CA, USA). In some experiments, mice were injected with a neutralizing anti-HMGB1 antibody (50 μg per mice) kindly provided by Huan Yang (Lexington, MA, USA). HMGB1 enzyme-linked immunosorbent assay II kits were obtained from SHINO-TEST CORPORATION (Tokyo, Japan). In some experiments, cells were exposed to recombinant CRT, at 3 μg per 10^6 cells in phosphate buffered saline (PBS) on ice for 30 min, as described earlier (Obeid *et al*., 2007b; Panaretakis *et al*., 2009), followed by three washes. Recombinant HMGB1 was purchased from R&D systems (Minneapolis, MN, USA), and 200 ng per mouse was injected along with dying tumor cells. Mitoxantrone (Sigma-Aldrich, St Louis, MO, USA), OXP (Sanofi Synthelabo, Paris, France) and CDDP (Sigma-Aldrich) were used to induce cell death.

Quantification of cell death

Cell death was assessed by annexin V fluorescein isothiocyanate (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) staining. Briefly, 2×10^5 cells per sample were collected, washed in PBS, pelleted, and resuspended in an incubation buffer (10 mM HEPES/NaOH (pH 7.4), 140 mm NaCl and 5 mM $CaCl_2$) containing annexin V fluorescein isothiocyanate antibody. Samples were kept in the dark and incubated for 15 min before the addition of another 400 μl of 0.1% propidium iodide incubation buffer and subsequent analysis on a fluorescence-activated cell sorter Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) using Cell Quest software.

Flow cytometric analysis of CRT on the cell surface

A total of 10^5 cells were plated in 12-well plates and treated the day after with the indicated agents for 4 h. Cells were collected, washed twice with PBS and fixed in 0.25% paraformaldehyde in PBS for 5 min. After washing again twice in cold PBS, cells were incubated for 30 min with primary antibody, diluted in cold blocking buffer (2% fetal bovine serum in PBS), followed by washing and incubation with the Alexa488-conjugated monoclonal secondary antibody in a blocking buffer (for 30 min). Each sample was then analysed by FACScan (Becton-Dickinson) to identify cell surface CRT. Isotype-matched IgG antibodies were used as a control, and the fluorescent intensity of stained cells was gated on propidium iodide-negative cells.

Immunofluorescence
For surface detection of CRT, cells were placed on ice, washed twice with PBS and fixed in 0.25% paraformaldehyde in PBS for 5 min. Cells were then washed twice in PBS, and a primary antibody, diluted in cold blocking buffer, was added for 30 min. After three washes in cold PBS, cells were incubated for 30 min with the appropriate Alexa 488-conjugated secondary antibody and the Alexa 633-conjugated wheat germ agglutinin (WGA 633) diluted in a cold blocking buffer. Cells were fixed with 4% paraformaldehyde for 20 min before nuclear staining with Hoechst 33342 (1/10000) in PBS for 20 min and then mounted on slides. For intracellular HMGB1 staining, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 (Sigma) for 10 min, rinsed three times with PBS, and nonspecific binding sites were blocked with 10% fetal bovine serum in PBS for 30 min. A primary antibody was added for 1 h. Subsequently, cells were washed three times with PBS and incubated for 30 min with an Alexa Fluor 488-conjugated secondary antibody (1:500, Molecular Probes, Carlsbad, CA, USA).

Detection of HMGB1 release
CT26, HCT116 and RKO cells were plated in 6-well plates with 2 ml full medium appropriate for the cell types. The medium was changed 24 h later and treatment was added onto the cells. Supernatants were collected at different time points, dying tumor cells were removed by centrifugation and supernatants were isolated and frozen immediately. Quantification of HMGB1 in the supernatants was assessed by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Small interfering RNA transfections
CT26 cells were transfected with small interfering RNA (siRNA) heteroduplexes specific for calreticulin (5′-rCrCrGr CUrGrGrGUrCrGrArAUrCrRrArATT-3′), HMGB1 (5′-rGr CrArGrCrCrCUrAUrGrArGrArArGrArArATT-3′ and rGrC UrGrArArArArGrArGrCrArArGrArArArATT) or an unrelated control (5′-rGrCrCrGrGUrAUrGrCrCrGrGUUrArAr GUTT-3′) designed in our laboratory and synthesized by Sigma-Proligo (St Louis, MO, USA), at a final concentration of 100 nM using HiPerFect (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. At 48 h after transfection, CT26 cells were assessed for total content of the respective proteins by immunobloting.

Anti-tumor vaccination
A total of 3×10^6 CT26 cells, untreated or treated with either OXP, CDDP or mitoxantrone for 4 h, were inoculated subcutaneously in 200 µl PBS into 6-week-old female BALB/c mice (Janvier, Charles River) into the lower flank, whereas 5×10^5 untreated control cells were inoculated into the contralateral flank 7 days later (Casares *et al*., 2005). To restore ecto-CRT, CT26 cells were incubated with recombinant CRT at 3 µg per 10^6 cells in PBS on ice for 30 min, followed by three washes. Animals that bore tumors in excess of 20–25% of the body mass or were necrotic, were killed. All animals were maintained in specific pathogen-free conditions, and all experiments were carried out according to the Federation of European Laboratory Animal Science Association guidelines. The Ethics Committee of Institut Gustave Roussy approved all the animal experiments.

Chemotherapy of established tumors in mice
Mice were injected in the flank with 5×10^5 CT26 cells. The tumor surface was monitored using calipers. When the tumor size reached 40–80 mm^2, mice were assigned into homogenous groups of 4–6 mice each treated with OXP or CDDP injected intratumorally in 50 µl PBS. Tumor volume was then calculated for comparison of tumor growth (Streit *et al*., 1999).

Priming assay
A total of 10^6 EG7 cells either untreated or treated with 300 µm OXP or 150 µM CDDP for 12 h (note that EG7 and CT26 cells differ in the kinetics of their response to OXP and CDDP) were injected into the footpad of the mice. After 5 days, gangliocytes of popliteal lymph nodes were collected and re-stimulated with OVA protein. After 72 h, interferon-γ secretion was assessed by enzyme-linked immunosorbent assay.

Clinical studies
The first clinical study analysed was the multicenter, prospective, controlled randomized trial designed to compare the efficacy of simplified LV5FU2 followed by FOLFOX6 to FOLFOX6 and by FOLFIRI on PFS after two lines of chemotherapy in metastatic colon cancer. A total of 410 patients were enrolled in this study with the following inclusion criteria:

Non-resectable metastases of histologically proven colorectal adenocarcinoma; Evaluable disease (World Health Organization (WHO) criteria: lesions >2 cm and outside of the irradiation field); Absence of previous chemotherapy other than adjuvant with 5-fluorouracil and folinic acid or concomitant to radiotherapy (rectum); WHO performance status lower or equal to 2.

The main end point of this study was PFS after two lines of chemotherapy. A total of 388 germline DNAs was prospectively obtained from whole blood samples of the 410 patients enrolled in this study.

The second clinical study analysed was the retrospective population-based registry study of non-metastatic colon cancer (Dukes stage II colon cancer) patients that were treated by surgical removal of the tumor, without any adjuvant treatment. A total of 258 stage-II colon cancer patients linked with a frozen tumor sample were included in the Cote d'Or digestive registry between 1997 and 2005, and were followed for progression 5 years after diagnosis. DNA was isolated from frozen blood leukocytes obtained from all subjects. PCR primers (Applied Biosystems, Villebon, France) 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 FAM signal to its corresponding VIC signal and calculating the log(FAM/VIC) ratio for each data point.

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 $\chi 2$ or Fisher's exact test, as appropriate. For clinical data, the log-rank test was used for the analysis of the Kaplan–Meier survival curves. A Cox model was then established to assess the role of *tlr4* variation among the different groups. Statistical analyses were performed with SAS software (SAS Institute Inc, Cary, NC, USA). All *P*-values were two tailed. A *P*-value of 0.05 was considered statistically significant for all experiments.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)