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# Activation of the NLRP3 inflammasome in dendritic cells induces IL-1β–dependent adaptive immunity against tumors

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**The therapeutic efficacy of anticancer chemotherapies may depend on dendritic cells (DCs), which present antigens from dying cancer cells to prime tumor-specific interferon-γ (IFN-γ)–producing T lymphocytes. Here we show that dying tumor cells release ATP, which then acts on $P2X_7$ purinergic receptors from DCs and triggers the NOD-like receptor family, pyrin domain containing-3 protein (NLRP3)-dependent caspase-1 activation complex ('inflammasome'), allowing for the secretion of interleukin-1β (IL-1β). The priming of IFN-γ–producing $CD8^+$ T cells by dying tumor cells fails in the absence of a functional IL-1 receptor 1 and in Nlpr3-deficient (*Nlrp3*$^{-/-}$) or caspase-1–deficient (*Casp-1*$^{-/-}$) mice unless exogenous IL-1β is provided. Accordingly, anticancer chemotherapy turned out to be inefficient against tumors established in purinergic receptor *P2rx7*$^{-/-}$or *Nlrp3*$^{-/-}$or *Casp1*$^{-/-}$hosts. Anthracycline-treated individuals with breast cancer carrying a loss-of-function allele of *P2RX7* developed metastatic disease more rapidly than individuals bearing the normal allele. These results indicate that the NLRP3 inflammasome links the innate and adaptive immune responses against dying tumor cells.**





It has been an ongoing conundrum to find which particular biochemical or metabolic alterations could be used to distinguish between immunogenic and nonimmunogenic cell death[1,2]. Physiological cell death, which occurs as a continuous byproduct of cellular turnover, is nonimmunogenic (or even tolerogenic). Avoidance of autoimmunity likewise results from the fact that physiological cell death fails to activate pattern recognition receptors, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs) that recognize pathogen and/or damage molecular patterns and activate innate immune effectors[3–9]. In contrast, cell death elicited by radiotherapy and some chemotherapeutic agents such as anthracyclines and oxaliplatin elicits an immune response required for the therapeutic success[10,11].

To mount a T cell immune response, DCs must incorporate antigens from stressed or dying cells, acquire the competence of antigen processing in a maturation step and present antigenic peptides bound to major histocompatibility complex (MHC) molecules in the context of co-stimulatory signals and cytokines that allow for the differentiation of specific T cells[12]. One of the peculiarities of immunogenic cell death is early, preapoptotic exposure of calreticulin on the plasma membrane, which facilitates the uptake of dying cells by DCs[13]. A second characteristic of immunogenic cell death is the release of high mobility group box-1 (HMGB1) protein from the nucleus into the surroundings of dying cells[14]. HMGB1 acts on TLR4 on DCs, and this interaction stimulates the processing of tumor antigens from dying cells[15]. However, addition of recombinant calreticulin or HMGB1 to live cancer cells is not sufficient to elicit the presentation of tumor antigens by DCs[16], implying that additional, yet-to-be-identified signals must be exchanged between dying cells and DCs.

In macrophages, the so-called inflammasome serves as a central sensor for pathogen and/or damage molecular patterns[17–19]. In response to danger signals, NLRP3 (also called NALP3 or cryopyrin) interacts with the adaptor molecule apoptosis-associated speck-like protein

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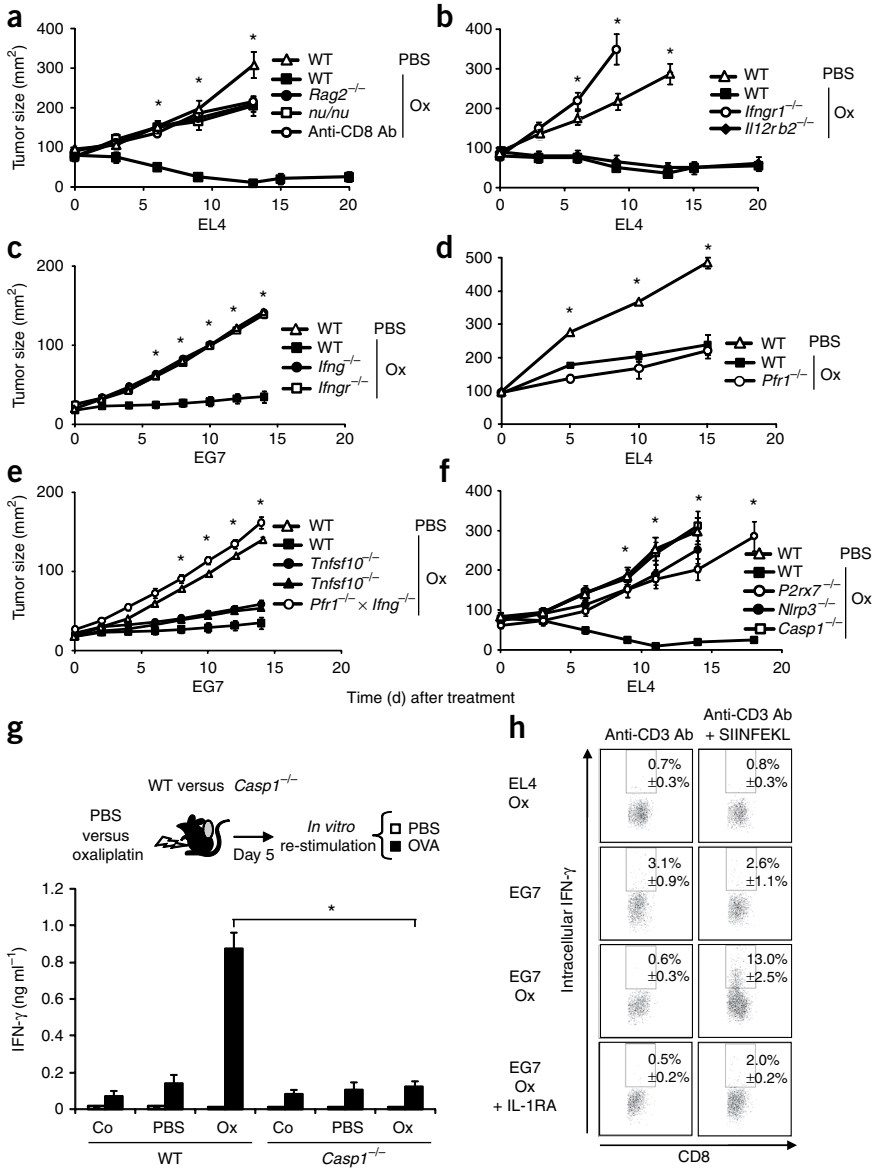


**Figure 1** The tumoricidal activity of oxaliplatin depends on the NLRP3 inflammasome. (**a**–**f**) EL4 or EG7 thymoma tumor size in syngeneic mice bearing the indicated genotypes (**a**–**f**) or injected with CD8-specific antibody (Anti-CD8 Ab) and treated with oxaliplatin (Ox) (**a**). Each treatment group included five or six mice (data are means ± s.e.m.), and each experiment was repeated at least twice with similar results. $^*P < 0.05$. PBS-treated controls are only shown for tumors growing on WT mice. For PBS-treated controls in all genotypes, see **Supplementary Figure 1**. *Pfr1*, gene encoding perforin. (**g**,**h**) IFN-γ secretion by inguinal lymph node cells draining established EG7 tumors treated or not with oxaliplatin (Ox) and re-stimulated with OVA, as measured by ELISA (**g**). Monitoring by immunofluorescence detection of intracellular IFN-γ of the proportion of IFN-γ–positive CD8$^+$ T cells in inguinal lymph nodes draining established EL4 or EG7 treated or not with Ox after *ex vivo* re-stimulation with antibody to CD3ε ± SIINFEKL peptides (**h**). Results (means of triplicates ± s.e.m., $n = 3$ per group) are representative of one typical experiment out of three. $^*P < 0.01$.

(ASC, encoded by the *Pycard* gene) to form the inflammasome, the principal Casp-1 activation complex[20–22]. Casp-1 activated by the NLRP3 inflammasome is required for the proteolytic maturation of pro–IL-1β and hence IL-1β secretion in response to purinergic $P2RX_7$ receptor agonists and TLR4 ligands[23–26]. Multiple distinct bacterial products or endogenous damage signals stimulate the NLRP3 inflammasome, resulting in the proteolytic activation of caspase-1[25,27–30]. Gain-of-function mutations in *NLRP3* that increase IL-1β secretion account for hereditary inflammatory diseases[31,32].

Here we report the unexpected finding that the activation of the NLRP3 inflammasome within DCs is decisive for the immune response against dying tumor cells, providing a previously unknown link between the innate and acquired immune systems.

## RESULTS

### Chemotherapy efficacy relies on the NLRP3 inflammasome

Oxaliplatin is efficient in controlling tumor growth in immunocompetent wild-type (WT) mice but loses its therapeutic efficacy on syngeneic EL4 thymomas in *Rag2*$^{-/-}$ mice, which lack T and B cells, in *nu/nu* mice, which lack T cells, in mice that have been depleted of CD8$^+$ T cells by injection of a specific antibody (**Fig. 1a**; for treated controls, see **Supplementary Fig. 1** and **Supplementary Methods**), and in mice deficient in IFN-γ receptor-1 (*Ifngr1*$^{-/-}$ mice), yet remains efficient in mice deficient in IL-12 receptor-β2 (*Il12rb2*$^{-/-}$ mice) (**Fig. 1b**). The gene-targeting of *Ifngr1 or Ifng* also abolished the therapeutic response against EG7 thymomas (EL4 expressing the model antigen ovalbumin, OVA) (**Fig. 1c**). In contrast, EL4 or EG7 thymomas fully responded to chemotherapy in mice lacking perforin or tumor necrosis factor–related apoptosis-inducing ligand (TRAIL, encoded by *Tnfsf10*; **Fig. 1d**,**e**). We obtained similar data indicating the requirement for IFN-γ (but not perforin or TRAIL) for the efficacy of chemotherapy for mouse CT26 colon cancers and mouse MCA2 sarcomas treated with doxorubicin (**Supplementary Fig. 2** and **Supplementary Methods**).

In addition, tumors implanted in mice that lack either of two components of the inflammasome[28], NLRP3 or Casp-1, failed to respond to oxaliplatin (**Fig. 1f**). Systemic oxaliplatin treatment primed T cells from the tumor-draining lymph node for IFN-γ production, and this chemotherapy-induced T cell priming was deficient in *Casp1*$^{-/-}$ mice (**Fig. 1g**). Intracellular staining revealed that dying tumor cells primed CD8$^+$ T cells for IFN-γ production and that an IL-1 receptor antagonist (IL-1RA[33]) applied with the chemotherapy abolished this T cell priming (**Fig. 1h**). Therefore, CD8$^+$ T cell–mediated, IFN-γ–dependent antitumor immune responses elicited by chemotherapy are controlled at some level by the NLRP3 inflammasome.

### ATP released from dying cells engages DC $P2RX_7$ receptors

One of the most pleiotropic activators of the NLRP3 inflammasome is extracellular ATP, which is released from stressed cells[34]. Oxaliplatin (and a variety of cytotoxic agents) induced the release

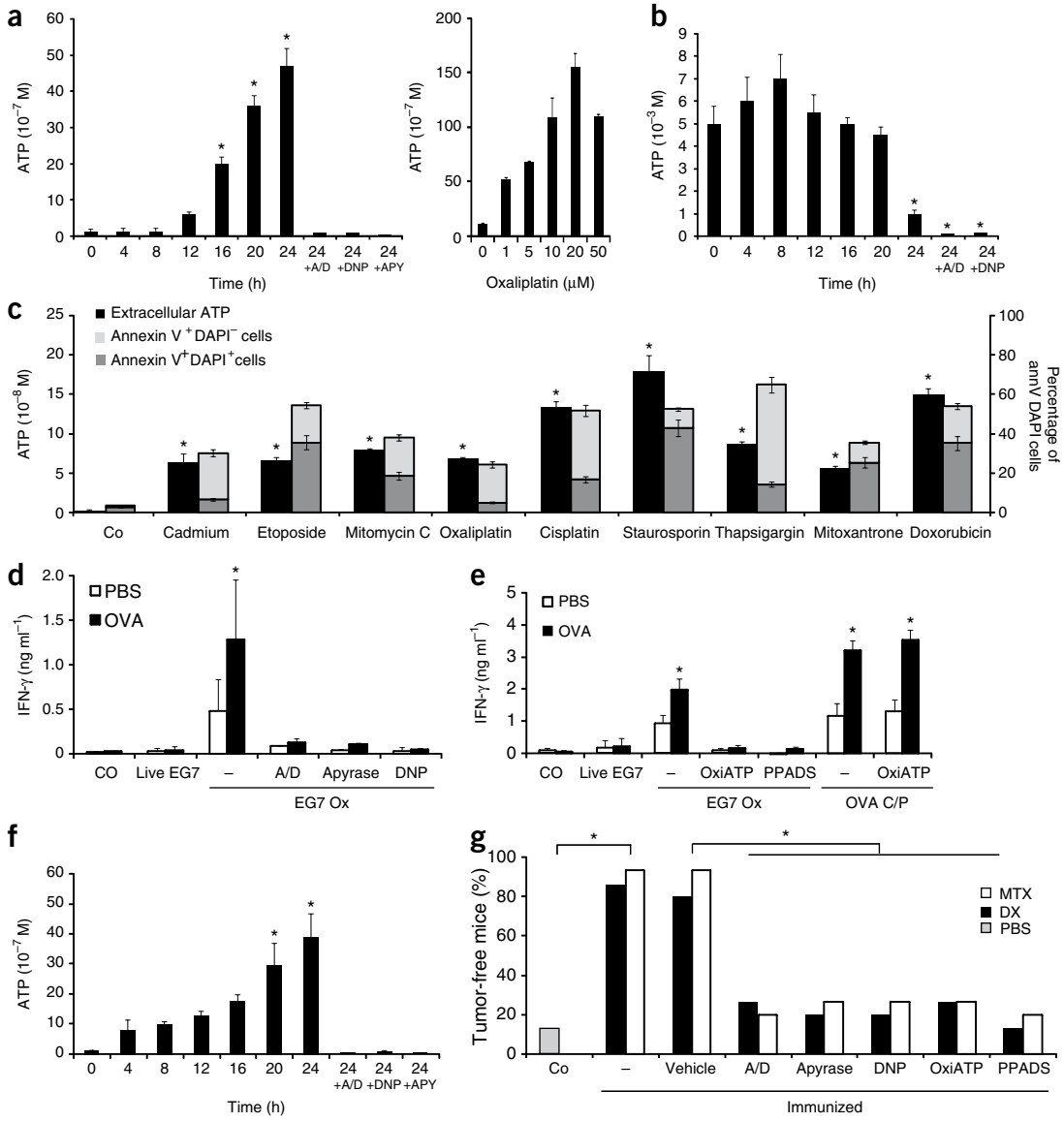


**Figure 2** ATP release by dying tumor cells dictates the immunogenicity of cell death. (**a**,**b**) ATP release into the supernatant (**a**) or residual intracellular ATP (**b**) in EG7 cells treated with oxaliplatin and with antimycin A plus deoxyglucose (A/D), DNP or apyrase (APY). Means ± s.e.m. of triplicates of one representative experiment out of three are depicted. The graph at right in **a** depicts the dose-response of ATP release monitored at 24 h. (**c**) Quantification of ATP released by EG7 cells treated with various cytotoxic agents using a selected dosage of each drug mediating cell death. $^{*}P < 0.01$. (**d**,**e**) Quantification of IFN-γ released by OVA-stimulated lymph node cells after immunization with ATP-depleted, oxaliplatin-treated EG7 cells (**d**) or Ox-treated EG7 admixed with oxidized ATP (oxiATP) or PPADS (**e**) using IFN-γ enzyme immunoassay. Means ± s.e.m. of three independent experiments are shown. (**f**) ATP release by CT26 cells exposed to doxorubicin. (**g**) Percentages of tumor-free mice rechallenged with live syngeneic CT26 cells after immunization with CT26 cells treated with mitoxanthrone or doxorubicin and ATP scavengers or inhibitors of P2RX receptors ($n = 15$ per group). One representative experiment out of three is shown.$^{*}P < 0.05$.

of ATP from EL4 cells (data not shown), as well as from EG7 cells (**Fig. 2a–c**). Simultaneous inhibition of glycolytic ATP generation (with deoxyglucose) and oxidative phosphorylation (with antimycin A) or uncoupling of mitochondria with 2,4-dinitrophenol (DNP) resulted in the depletion of ATP from EG7 cells (**Fig. 2b**) without the induction of necrosis (data not shown). ATP depletion or addition of the ATP-degrading enzyme apyrase abolished the capacity of dying EG7 cells to trigger a local immune response after oxaliplatin treatment. After injection into the footpad, ATP-containing (but not ATP-depleted), oxaliplatin-treated EG7 cells primed T cells from the popliteal lymph node to secrete IFN-γ in response to re-stimulation with OVA (**Fig. 2d**). Blockade of purinergic receptors with the 2′,3′-dialdehyde derivative of ATP ('oxidized ATP') or with PPADS (iso-pyridoxalphosphate-6-azophenyl-2′,5′-disulphonate, which inhibits the P2X subclass of purinergic receptors) abolished T cell priming by DCs exposed to oxaliplatin-treated EG7 cells but not to OVA protein (**Fig. 2e**).

We obtained similar results for CT26 mouse colon cancer cells, which released ATP in response to doxorubicin (**Fig. 2f**). Blockade of purinergic receptors or depletion of ATP (**Fig. 2f**) from doxorubicin- or mitoxanthrone-treated CT26 cells abolished their capacity to protect the host against a lethal rechallenge with CT26 (**Fig. 2g**). Moreover, oxaliplatin-treated EG7 cells failed to prime T cells for IFN-γ production when they were inoculated into the

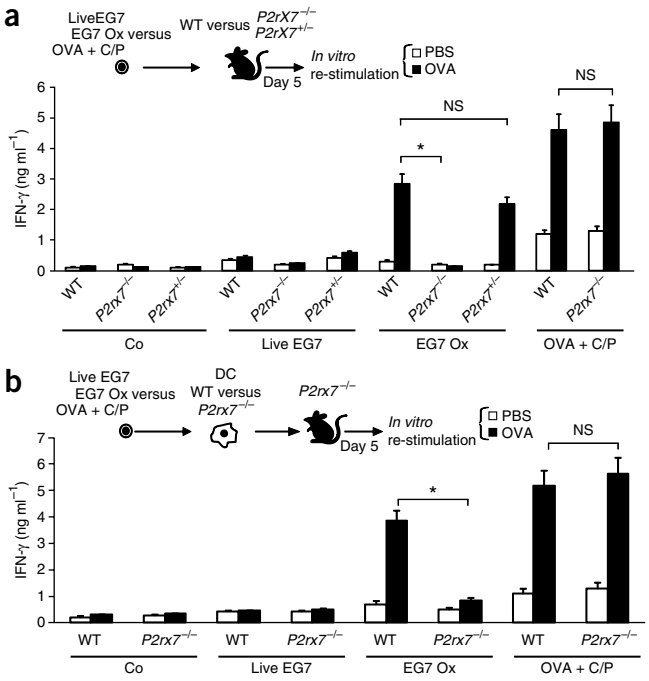


**Figure 3** Purinergic $P2RX_7$ receptors are mandatory for the DC-mediated immunogenicity of cell death. (**a**) Quantification of IFN-γ secretion using ELISA in popliteal lymph node cells restimulated with OVA after footpad inoculation of live versus oxaliplatin-treated EG7 cells in C57BL/6 mice (WT or *P2rx7*$^{+/-}$ or *P2rx7*$^{-/-}$). In parallel, mice were injected with 1 mg of OVA protein plus 10 μg CpG 28 and 5 μg polyI:C (C/P) as adjuvant. NS, not significant. (**b**). Same experiment as in **a**, but recipient mice were *P2rx7*$^{-/-}$ mice treated with an adoptive transfer of WT or *P2rx7*$^{-/-}$ DCs loaded with antigen (OVA holoprotein with C/P adjuvant, live or oxaliplatin-treated EG7 cells). The experiments included three or four mice per group and were repeated three times with similar results. *$P < 0.05$.

footpad of *P2rx7*$^{-/-}$ mice (ref. 35), in conditions in which they mediated T cell priming in WT *P2rx7*$^{+/+}$ or heterozygous *P2rx7*$^{+/-}$ controls (**Fig. 3a**). Bone marrow–derived DCs from WT mice (but not from *P2rx7*$^{-/-}$ mice) could prime T cells when they were preincubated with dying EG7 cells and then inoculated into *P2rx7*$^{-/-}$ mice (**Fig. 3b**), indicating that it is indeed the $P2RX_7$ receptor on DCs that senses ATP from dying tumor cells as an obligate co-signal of immunogenicity. EL4 tumors implanted in *P2rx7*$^{-/-}$ mice responded less efficiently to oxaliplatin than tumors growing in WT mice (**Fig. 1f**), underscoring the importance of $P2RX_7$ for chemotherapeutic responses.

### Dying tumor cells activate the NLRP3 inflammasome in DCs

DCs incubated with dying EG7 cells activated caspase-1, as determined by immunofluorescence staining with an antibody that recognizes a neoepitope formed by proteolytic maturation of caspase-1 (**Fig. 4a**,**b**). Caspase-1 activation, as observed in WT DCs, was inhibited by oxidized ATP (**Fig. 4a**,**b**). DCs from *P2rx7*$^{-/-}$, *Pycard*$^{-/-}$ (ref. 36) or *Nlrp3*$^{-/-}$ mice were unable to activate caspase-1 (**Fig. 4a**,**b**). The proteolytic maturation of caspase-1 strictly correlated with the caspase-1–dependent secretion of IL-1β (but not IL-18, data not shown). Thus, in contrast to WT DCs, *P2rx7*$^{-/-}$, *Pycard*$^{-/-}$ or *Nlrp3*$^{-/-}$ DCs failed to secrete IL-1β in response to dying tumor cells, and oxidized ATP efficiently suppressed IL-1 β secretion by WT DCs in these conditions (**Fig. 4c**). The acquisition of MHC class II antigen, CD40, CD80 or CD86 molecules by DCs stimulated with dying tumor cells was independent of NLRP3 or Casp1 (data not shown), and the production of the IL-12 p40 subunit by DCs exposed to dying tumor cells was not affected by the knockout of *P2rx7*, *Nlrp3* or *Pycard* (**Fig. 4d**). Accordingly, *Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$ DCs presented the OVA-derived SIINFEKL peptide to T cell hybridoma cells as efficiently as WT DCs (**Supplementary Fig. 3** and **Supplementary Methods**).

### Priming of CD8$^+$ cells requires a functional NLRP3 inflammasome

The OVA-specific IFN-γ production elicited by dying EG7 cells was abolished in *Nlrp3*$^{-/-}$, *Casp1*$^{-/-}$ and mice deficient in the IL-1 receptor-1 (but normal in *Il12rb2*$^{-/-}$ and *Il18r1*$^{-/-}$ mice) (**Fig. 5a**). Moreover, dying CT26 cells primed T cells for IFN-γ production in response to autologous CT26 lysate (but not control lysates from a distinct tumor) (**Supplementary Fig. 4a**). This tumor-specific response was abolished by injecting IL-1RA (**Supplementary Fig. 4a**). Cross-priming of MHC class I–restricted CD8$^+$ T cells in an MHC-incompatible system also depended on caspase-1 because dying mouse embryonic fibroblasts expressing cell-associated OVA (fused to the transmembrane domain of MHC $K^b$ protein) primed T cells only when injected into WT (but not *Casp1*$^{-/-}$) mice (**Fig. 5b** and **Supplementary Fig. 4b**,**c**). DCs from *Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$ mice were unable to prime T cells to respond to OVA when they were first incubated with oxaliplatin-treated EG7 cells and then injected into the footpad of normal mice (**Supplementary Fig. 5**).

Similarly, oxaliplatin-treated B16F10 melanoma cells (which express the endogenous tumor antigen gp100) induced gp100-specific, IFN-γ–producing CD8$^+$ T cells in WT, but not in *Casp1*$^{-/-}$, mice (**Fig. 5c**). In yet another tumor model, mitoxantrone-treated mouse MCA205 fibrosarcoma cells elicited a protective immune response that prevented the growth of live tumor cells in WT mice but not in mice deficient in *P2rx7*, *Nlrp3* or *Casp1* (**Fig. 5d**). Neutralizing antibodies to IL-1R1 or IL-1β (but not IL-1α) also blunted the therapeutic response of two independent transplantable methylcholanthrene (MCA)-induced sarcomas to doxorubicin (**Fig. 5e**) and that of CT26 cells to anthracyclines or oxaliplatin *in vivo* (data not shown). Notably, the IL-1β–dependent efficacy of doxorubicin could also be shown in a proportion (8 of 15) of primary fibrosarcomas elicited by MCA treatment *in vivo*[37] in C57BL/6 mice (**Fig. 5f**) and BALB/c mice (data not shown). Together, these results suggest that the NLRP3 inflammasome–mediated caspase-1 activation in DCs is stringently required for an IL-1β–dependent antitumor T cell response.

### IL-1β secretion by DCs is mandatory for T cell priming

To directly assess the contribution of IL-1β to the priming of T cells, we performed *in vitro* priming assays using bone marrow–derived DCs (derived from WT or *Casp1*$^{-/-}$ mice) that were pulsed with dying tumor cells and then cultured with naive T cells in the presence of IL-1RA or antibodies that neutralize IL-1β. MHC H-$2^b$–expressing *Casp1*$^{-/-}$ DCs incubated with oxaliplatin-treated EG7 cells failed to prime naive T cells from OT-1 mice, which express a transgenic T cell receptor that recognizes the OVA-derived SIINFEKL peptide presented by H-$2^b$ class I molecules (**Fig. 6a**). In similar conditions, WT DCs did stimulate OVA-specific OT-1 cells to produce IFN-γ, and this effect was inhibited by addition of IL-1β–specific antibodies (**Fig. 6a**). Conversely, exogenous recombinant IL-1β (rIL-1β) or rIL-12 restored the priming capacity of *Casp1*$^{-/-}$ DCs *in vitro*

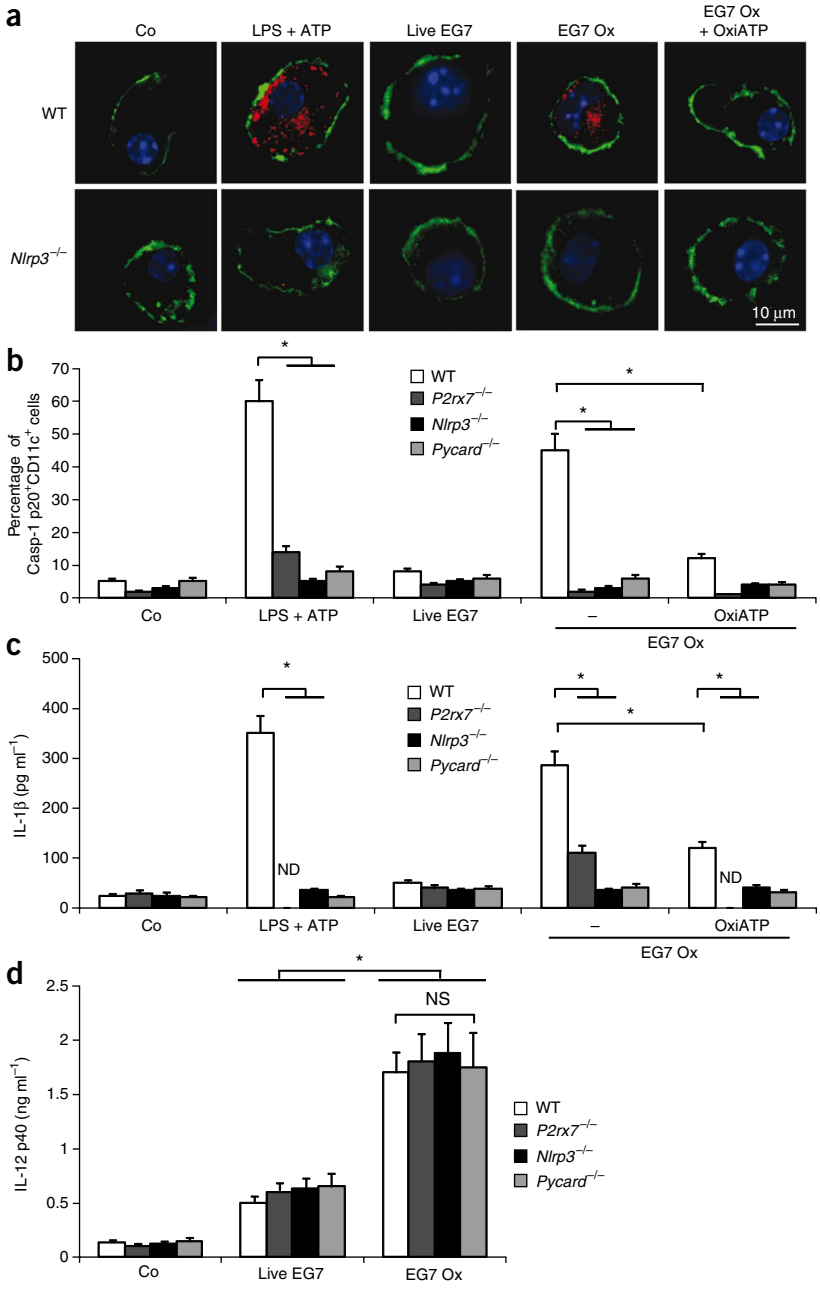


**Figure 4** Activation of caspase-1 and IL-1β secretion in DCs exposed to dying tumor cells. (**a**,**b**) Immunofluorescence staining of active caspase-1 in bone marrow–derived DCs (**a**) and quantification of DCs containing active caspase-1 (**b**). DCs (WT or derived from *P2rx7*$^{-/-}$, *Nrlp3*$^{-/-}$ or *Pycard*$^{-/-}$ mice) were exposed to PBS or oxaliplatin or to live or oxaliplatin-treated EG7 tumor cells in the presence or absence of oxidized ATP and labeled with antibody to CD11c, antibody to caspase-1 p20 and DAPI (**a**,**b**). Positive controls included DCs exposed to LPS (50 ng ml$^{-1}$) for 16 h and then to ATP (2 mM) for 20 min. (**c**,**d**) IL-1β (**c**) or IL-12 p40 (**d**) concentrations measured by commercial ELISA in the supernatants of DCs. ND, not determined. The experiments were performed three times with similar results. Representative microphotographs or histograms are depicted. Means ± s.e.m. are shown for triplicate wells. *$P < 0.05$.

(**Fig. 6a**). It is noteworthy that OT-1 activation was promoted by WT and *Casp1*$^{-/-}$ DCs with similar efficacies if the DCs were stimulated by soluble OVA protein and TLR3 and TLR9 agonists instead of dying OVA-expressing cells (**Fig. 6a**). Upon culturing with WT DCs (but less so with *Casp1*$^{-/-}$ DCs) pulsed with dying cells, OT1 cells upregulated messenger RNA specific for IFN-γ (but not for perforin) (data not shown). To show a direct role of IL-1β in stimulating CD8$^+$ T cells, we stimulated CD3$^+$CD8$^+$ splenic T cells by simultaneously cross-linking CD3 and CD28 in the absence or presence of IL-1β. Naive CD8$^+$ T cells (which express basal amounts of CD121a, the IL-1β receptor, **Fig. 6b**) failed to produce IFN-γ, unless we added rIL-1β (or rIL-12 as a positive control, but not tumor necrosis factor-α or IL-6 as negative controls) (**Fig. 6b**). Oxaliplatin-treated EG7 cells failed to prime OVA-specific T cells *in vivo* after inoculation into the footpad if they were injected along with IL-1RA (**Fig. 6c**). Moreover, *in vitro*–re-stimulated CD3$^+$CD8$^+$ T lymphocytes failed to stain positively for cytoplasmic IFN-γ in WT mice injected with IL-1RA together with oxaliplatin-treated EG7 cells, or in *Casp1*$^{-/-}$ mice (**Fig. 6d**). Accordingly, IL-1RA blocked the expansion of antigen-specific CD8$^+$ T cells that were induced by dying EG7 (but not EL4) cells *in vivo* and then were detected with a SIINFEKL-K$^b$ tetramer *ex vivo* (**Fig. 6e**). The P2RX$_7$–NLRP3–Casp-1–dependent antitumor effect also required CD4$^+$ T cell help (**Supplementary Fig. 6a**,**b**).

Immunization with oxaliplatin-treated EG7 cells promoted T helper type 1 polarization. Expression of CD69 and production of IFN-γ and IL-2 by CD4$^+$ T cells located in the draining lymph node occurred in a Casp-1–dependent manner (**Supplementary Fig. 6c**,**d**). In mice bearing a deficient P2RX$_7$–NLRP3–Casp-1 axis, the oxaliplatin-elicited OVA-specific response was shifted to a T helper type 2 pattern (with production of IL-10 and IL-13) (**Supplementary Fig. 7**). These results underscore the cardinal contribution of IL-1β to the immune response elicited by dying tumor cells. In agreement with this interpretation, local injection of recombinant IL-1β protein (or IL-12, but not IL-6), together with oxaliplatin-treated EG7 cells, fully restored deficient T cell priming in *Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$ mice (**Fig. 6f**). Thus, IL-1β is the factor that determines the NLRP3-dependent immunogenicity of cancer cell death.

### *P2RX7* mutations in human breast cancer

Loss-of-function alleles of the gene encoding TLR4, a receptor involved in the perception of immunogenic cell death, negatively affect the prognosis of individuals with breast cancer treated with anthracyclines[15]. We sought to determine whether a loss-function polymorphism that affects P2RX$_7$ (Glu496Ala, rs3751143), lowering its affinity for ATP[38] and thus IL-1β release in human monocytes[39], would decrease the

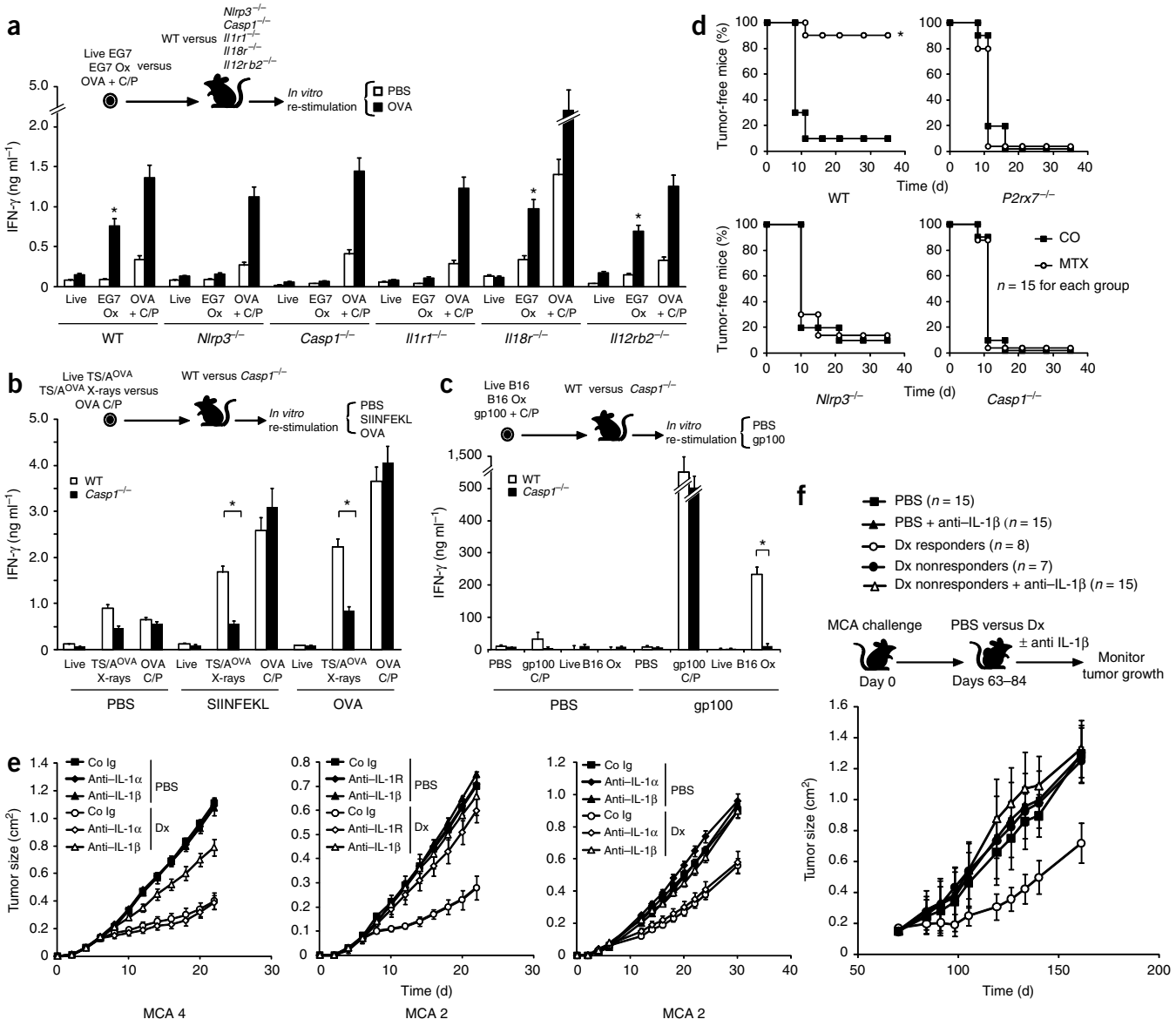


**Figure 5** The NLRP3 inflammasome is required for the elicitation of an adaptive antitumor immune response. (**a**–**c**) Quantification of IFN-γ secretion, using ELISA, by lymph node cells re-stimulated with SIINFEKL peptides (**b**) with or without OVA holoprotein (**a**,**b**) or with gp100-derived peptide (QVPRNQDWL) (**c**) after immunization of MHC H-2$^{b+}$ WT (**a**–**c**), *Nlrp3*$^{-/-}$ (**a**), *Casp1*$^{-/-}$ (**a**–**c**) or *IL1r1*$^{-/-}$ (**a**) mice (five mice per group) with oxaliplatin-treated EG7cells (**a**),irradiated H-2$^{d+}$ mouse mammary cancer TS/A cells (**b**) or oxaliplatin-treated B16F10 cells (**c**). (**d**) Percentages of tumor-free mice after rechallenge with syngeneic MCA205 sarcoma after immunization with mitoxantrone (MTX)-treated MCA205 cells in the indicated genetic backgrounds of C57BL/6 mice ($n = 15$ per group). (**e**) Tumor growth kinetics of day 7–established MCA-2 or MCA-4 sarcomas treated with doxorubicin (Dx) in the presence of control immunoglobulin (Co Ig), neutralizing antibody to IL-1α (Anti–IL-1α), IL-1β (Anti–IL-1β) or IL-R1 (Anti–IL-1R1) ($n = 5$ per group). (**f**) Tumor growth kinetics of spontaneous MCA-induced sarcomas in C57BL/6 mice treated with intravenous doxyrubicin with or without neutralizing antibodies to IL-1β ($n = 15$ per group). Tumor growth kinetics of one typical experiment (**e**) from six different MCA lines assessed or one experiment (**f**) separating responders from nonresponders are depicted.

beneficial effect of anthracyclines in breast cancer. We analyzed a cohort of individuals with sporadic breast cancer that was stratified according to the *P2RX7* genotype (normal versus variant *P2RX7*). The frequencies of heterozygous ('variant') and homozygous ('normal') germline polymorphisms were 36% and 64%, respectively. We found no significant differences in classical prognostic factors between normal and variant group of patients (**Supplementary Table 1**). However, the *P2RX7* loss-of-function allele had a significant negative prognostic impact on metastatic disease-free survival (log-rank test; $P = 0.02$).

The univariate Cox regression analysis confirmed statistically significant results for the *P2RX7* genotype ($P = 0.021$), with a hazard ratio of 1.6 (95% confidence interval [1.074; 2.398]) for *P2RX7* Glu496Ala versus *P2RX7* Glu496Glu (**Fig. 6g** and **Supplementary Table 1**). A multivariate Cox regression model revealed a significant effect for tumor grade (tumor grade 1 versus tumor grade 2–3, hazard ratio 3.243[1.024;4.383], $P < 0.05$) and for *P2RX7* genotype (Glu496Glu versus Glu496Ala, hazard ratio 1.556[1.017;2.383], $P < 0.05$). These results lend support to the clinical relevance of the $P2RX_7$ and inflammasome pathway.

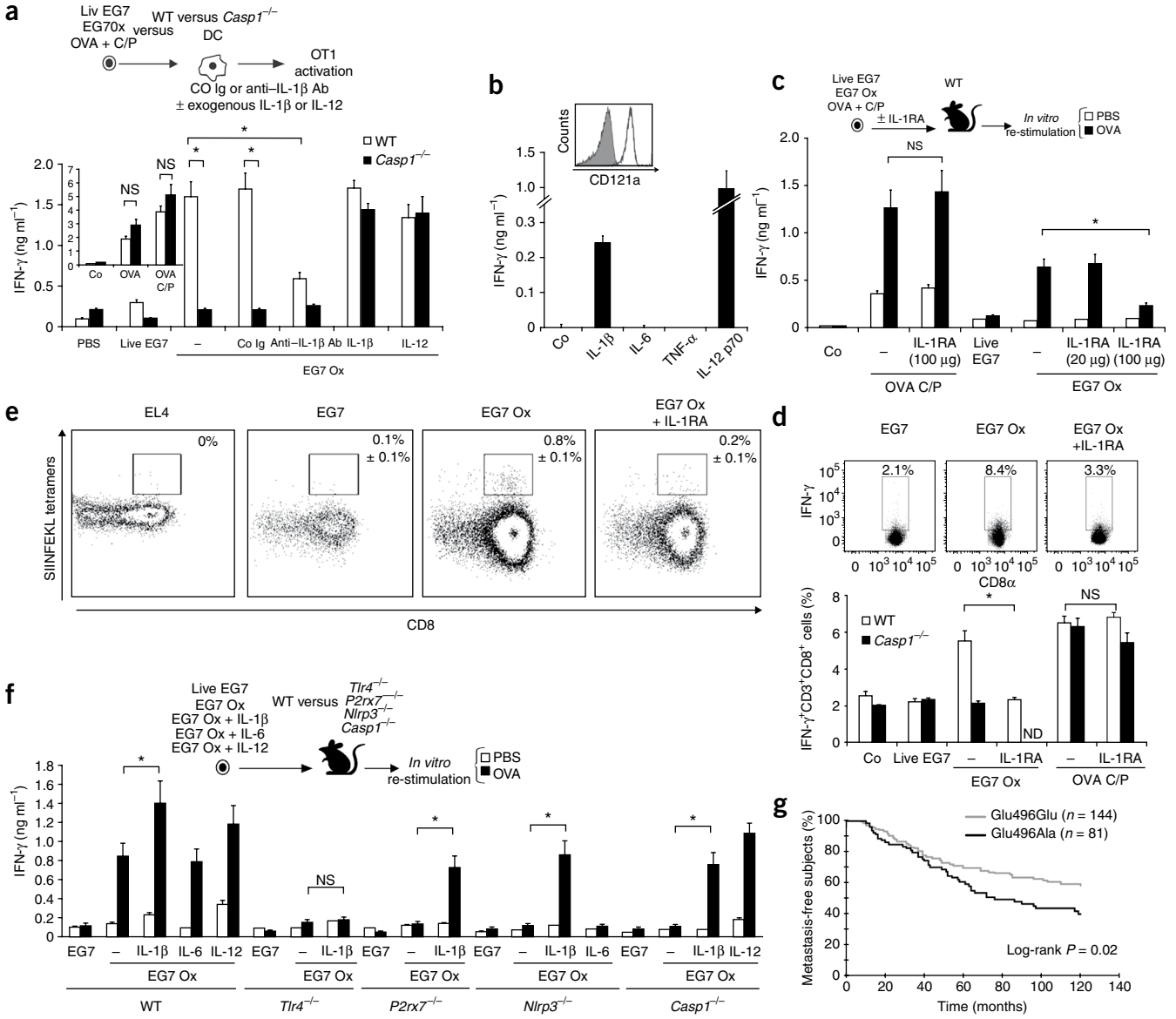


**Figure 6** NLRP3 inflammasome-dependent differentiation of tumor specific CD8+ T cells toward IFNγ polarization. (**a**,**b**) Monitoring of IFN-γ concentrations in supernatants of a 4-d coculture of naive OT-1 cells (**a**) with syngeneic WT or *Casp1*$^{-/-}$ bone marrow–derived DCs loaded with oxaliplatin-treated EG7 or OVA in C/P (inset) in the presence of neutralizing monoclonal antibody to IL-1β or exogenous rIL-1 (100 ng ml$^{-1}$) or IL-12 (10 ng ml$^{-1}$). Purified CD3+CD8+ T cells (**b**) were stimulated for 72 h with plastic-immobilized monoclonal antibodies to CD3 and CD28 with cytokines (10 ng ml$^{-1}$ for IL-12 p70, 100 ng ml$^{-1}$ for others). Inset shows CD121a (IL-1R1) expression on naive CD8+ T cells (isotype control in gray). (**c**) Quantification of IFN-γ produced by lymph node cells re-stimulated with OVA after immunization with oxaliplatin (Ox)-treated EG7 cells in the presence of IL-1RA molecules. (**d**) The frequency of IFN-γ–producing CD8+ T cells after re-stimulation of the lymph node is shown (representative dot plots at the top, means ± s.e.m. ($n = 3$) at the bottom). (**e**) The frequency of CD8+ T cells staining positively with SIINFEKL-K$^b$ tetramers in the draining lymph node of oxaliplatin-treated EG7 or EL4 cells determined at day 5. (**f**) Quantification of IFN-γ produced by lymph node cells re-stimulated with OVA after immunization with oxaliplatin-treated EG7 cells in the presence of the indicated cytokines in the indicated mouse backgrounds. *$P < 0.05$. (**g**) Kaplan-Meier estimates of time to metastasis in two groups of subjects with breast cancer treated with adjuvant anthracyclines ($n = 225$) bearing the normal (Glu496Glu) or loss-of-function (Glu496Ala) *P2RX7* alleles.

## DISCUSSION

Our results identify a new signal that is required for cancer cell death to be immunogenic, namely the release of ATP. This signal has been previously shown to increase as a danger signal in severe tissue damage including myocardial infarction and hepatotoxic insult[18], with potent proinflammatory effects. Because most cells express ATP-degrading ectoenzymes[40], ATP released from stressed or dying cells likewise follows a steep gradient and only activates purinergic receptors in the immediate vicinity of the lethal event. Our data are compatible with a scenario in which ATP activates $P2RX_7$ receptors on DCs, thereby stimulating the aggregation of the NLRP3-ASC–Casp-1 inflammasome, the proteolytic maturation of caspase-1, pro–IL-1β cleavage and consequent IL-1β release. IL-1β then is required for the priming of IFN-γ–producing, tumor antigen–specific CD8+ T cells. In accord with previous studies addressing the capacity of adoptively transferred or vaccine-induced CD8+ T cells to eradicate established tumors[41,42], IFN-γ, rather than cytolytic activities (that are dependent on perforin or TRAIL), mediates the anticancer activity of T lymphocytes

that have been primed in a $P2RX_7$–NLRP3–ASC–Casp-1–IL-1β–dependent fashion.

Each of the individual steps in this linear cascade (ATP to $P2RX_7$ to NLRP3–ASC–Casp-1 to IL-1β to $CD8^+$ T cell polarization for IFN-γ production) have been described previously[18,25,29,43,44]. However, there are key notions that emerge from our study. First, it was unexpected that, when activated in DCs, inflammasome activation can establish a link between the innate (inflammatory) and the acquired (cognate) immune responses because IL-1β produced by DCs is required for the priming of T cells. Second, none of the functional entities of this cascade (the ATP-$P2X_7$ system, the inflammasome and the IL-1β–IL-1βR axis) has previously been described as involved in anticancer immune responses that determine the efficacy of chemotherapy.

The NLRP3 inflammasome responds to multiple danger signals, including a decrease in cytosolic potassium concentrations and an increase in cytosolic DNA levels, urate and bacterial products[24,45–47]. The NLRP3 inflammasome is also controlled by the serial ligation of purinergic receptors and TLRs[18,23]. In addition to tumor cell–derived ATP acting on $P2RX_7$ receptors, HMGB1, a TLR4 agonist released by dying tumor cells treated with doxorubicin or oxaliplatin[15], may contribute to the stimulation of the NLRP3 inflammasome. Indeed, our data indicate that HMGB1 could synergize with ATP to induce IL-1β release by DCs (**Supplementary Fig. 8a**) and that antibody specific for HMGB1 abrogated the ability of DCs to produce IL-1β in contact with dying tumor cells (**Supplementary Fig. 8b**). Hence, both ATP and HMGB1 released from dying cells contribute to the activation of the NLRP3 inflammasome in DCs.

Notably, not only injured tumor cells but also stressed healthy tissues may elicit a Casp-1–dependent T cell response. Thus, we observed that immortalized, nontransformed mouse embryonic fibroblasts expressing cell-associated OVA and oxaliplatin-treated peritoneal macrophages pulsed with OVA protein both triggered OVA-specific immune responses in a Casp-1–dependent manner. However, anticancer chemotherapy does not elicit a generalized autoimmune response, presumably because tumor cells die more efficiently than healthy cells, because they express a set of tumor-specific antigens against which no self-tolerance has been established, or both.

Although IL-1β could be instrumental at the level of DCs (by contributing to DC activation and migration), our data indicate that maturation of DCs triggered by dying cells is caspase-1 independent (data not shown), that IL-1β does not affect the capacity of DC to process cell-associated antigens and that IL-1β can directly act at the transcriptional level on T cells to polarize $CD8^+$ T cell immune responses *in vitro*.

Chronic inflammation including overactivation of the IL-1β–IL-1βR system[48] has been considered a tumor-promoting condition, arguing in favor of IL-1β inhibition for tumor prevention or therapy[49–52]. However, as shown here, the production of IL-1β by DCs and the action of IL-1β on specific $CD8^+$ T cells may contribute to therapeutically relevant anticancer immune responses.

The clinical study reported here underscores the medical relevance of the ATP-$P2RX_7$ pathway for anthracycline treated breast cancer individuals. These results may therefore have major implications by defining new constitutional markers of chemotherapy efficacy in cancer patients. It will be crucial to determine which chemotherapies have the capacity to induce immunogenic cell death and which type of tumors can actually generate immunogenic signals after radiotherapy or chemotherapy in humans.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

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


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### AUTHOR CONTRIBUTIONS

F.G., L. Aymeric., A.T., L. Apetoh., T.P., F.S., G.M., E.U., Y.M., N.M.M., N.M.H. and M.J.S. performed *in vitro* and *in vivo* experiments. C.O., E.T., P.G. and A.C. performed *in vitro* experiments. A.T. and T.P. performed immunofluorescence experiments. M.U., J.-L.P., B.R., J.K. and J.T. provided transgenic cells or mice and gave scientific advice. K.V., F.A., R.L., F.G. and A.T. performed the single nucleotide polymorphism analysis on cohorts of subjects with cancer. F.G., L. Apetoh., M.J.S. and A.T. prepared the figures and drafted the manuscript. G.K. and L.Z. designed the study and wrote the manuscript. M.J.S., G.K. and L.Z. all contributed equally to the design of the experiments and to the writing of the manuscript.

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## ONLINE METHODS

**ATP assays.** We treated $1 \times 10^5$ EG7 or CT26 cells (American Type Culture Collection) with oxaliplatin (5 μg.ml$^{-1}$), doxorubicin (20 μM) or other cell death inducers (all from Sigma-Aldrich), and measured intracellular or extracellular ATP at the time points indicated in **Figure 2**. Briefly, we collected supernatants and assessed the amount of extracellular ATP with the luciferin-based ENLITEN ATP Assay (Promega). We added 100 μl of luciferin-luciferase solution to the supernatants and recorded light emission with a Fluostar luminometer (BMG Labtech). We calibrated light emission by using standard samples furnished by the manufacturer. We measured intracellular ATP with a commercial ATP Assay kit (Calbiochem). We treated cells with a lysis solution containing 1% trichloroacetic acid for 1 min. We then added the luciferin-luciferase solution and measured ATP. For ATP depletion, we first treated cells with either oxaliplatin or doxorubicin for 24 h and then incubated them with antimycin A (2 μg ml$^{-1}$) and 2-deoxyglucose (30 mM) (Sigma Aldrich) for 20 min, and we subsequently measured ATP as described above. Alternatively, we treated the cells with DNP (200 μM) or apyrase (40 international units per ml) (Sigma Aldrich), for 20 min.

**Chemotherapy and radiotherapy of tumors established in mice.** We injected WT C57BL/6 mice or knockout mice subcutaneously with $1 \times 10^6$ EL4 (or EG7) cells into the right flank. Alternatively, we injected WT BALB/c mice with $5 \times 10^5$ CT26 cells or $1 \times 10^5$ MCA2 or MCA4 cells subcutaneously into the right flank. We then randomly assigned mice to treatment groups of four to six mice each. When tumor size reached 70–90 mm$^2$ for EL4 (9–12 d after injection), 15–25 mm$^2$ for EG7 (7 d after injection), 60–80 mm$^2$ for CT26 (8–10 d after injection) or 15–20 mm$^2$ for MCA2 and MCA4 (7 d after injection), we gave the mice chemotherapy. We injected mice bearing EL4 tumors with oxaliplatin (5 mg per kg body weight intraperitoneally), mice bearing CT26 tumors with oxiplatin (5 mg per kg body weight intraperitoneally) or doxorubicin (50 μl, 4 mM intratumorally) and mice bearing MCA2 or MCA4 tumors with doxorubicin (2 mg per kg body weight intravenously). In some experiments, CT26 tumor-bearing mice received two injections (on day 0 and day 2 after the first injection of chemotherapeutic treatment) of IL-1RA (100 μg), IL-1–specific antibody (100 μg) (R&D Systems, clone 30311) or hamster serum as a control. Some CT26 and MCA2 or MCA4 tumor–bearing mice received biweekly administration of control immunoglobulin, antibody to IL-1α, antibody to IL-1β or antibody to IL-1R (provided by R. Schreiber) from day 4 to day 21 after tumor inoculation. In individual mice where fibrosarcomas were generated *de novo* by MCA, treatment with PBS or doxorubicin in the presence of control or IL-1β–specific antibody began on first palpation of tumors >0.1 cm$^2$ in size. We administered doxorubicin twice, with injections 1 week apart (2 mg per kg body weight intravenously), and administered antibody twice weekly (250 μg intraperitoneally). Mice were bred and maintained according to both the Federation of European Laboratory Animal Science Associations and the Val de Marne Animal Experimental Ethics Committee guidelines.

***In vitro* stimulation of CD8$^+$ T cells or OT-1 cells by dendritic cells.** We purified CD8$^+$ T cells from OT-1 spleens with a CD8$^+$ T Cell Isolation Kit (Miltenyi Biotec). We cultured these responder cells ($2 \times 10^4$) with bone marrow–derived DCs from WT or Casp-1–deficient mice ($1 \times 10^4$) loaded with live or oxaliplatin-treated EG7 cells ($1 \times 10^4$) in the presence of a control or IL-1β–specific antibody (10 μg ml$^{-1}$) in round-bottom 96-well plates. Alternatively, we incubated OT-1 cells and bone marrow–derived DCs with OVA protein alone or with CpG (10 μg ml$^{-1}$) and polyI:C (5 μg ml$^{-1}$) (Sigma Aldrich). We collected the supernatants after 48 h and assayed them for IFN-γ by ELISA (BD Biosciences). Alternatively, we isolated double-positive CD3$^+$ and CD8$^+$ cells from mouse spleen by cell-sorting on a MoFlo cytofluorometer (Dako) and incubated them in flat-bottom 96-well culture plates precoated with monoclonal antibodies to CD3ε (1 μg ml$^{-1}$) (eBioscience, clone 145-2C11) and CD28 (0.5 μg ml$^{-1}$) (BD Biosciences, clone 37.51) supplemented with various amounts of cytokines. We collected the supernatants after 48 h and assayed them for IFN-γ by ELISA.

**Genotyping of P2RX7 single nucleotide polymorphism.** We obtained written informed consent from subjects entering the study approved by the local Ethical Committee of Kremlin Bicêtre and the Institut Gustave Roussy Biopathology Review Board. We isolated DNA from frozen blood leukocytes of each individual. We used Taqman primers and probe (Applied Biosystems) to amplify a fragment containing the *P2RX7* Glu496Ala single nucleotide polymorphism (rs3751143). After PCR amplification, we assigned genotypes 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.

**Additional methodology.** Specific reagents, tumor cell lines, mouse strains, primary DC cultures, anticancer prophylactic vaccines, immunofluorescence assays for Casp-1 activation, monitoring tumor-specific T cell responses and the design of the clinical study are all detailed in the **Supplementary Methods**.

**Statistical analyses.** We compared continuous data by the Mann-Whitney *U* test and compared categorical data by Chi-square or Fisher's exact test, as appropriate. We used the log-rank test for the analysis of Kaplan-Meier survival curves. We performed statistical calculations with JMP 5.1 software (SAS Institute). All *P* values were two-tailed. A $P$ value $< 0.05$ was considered statistically significant for all experiments.