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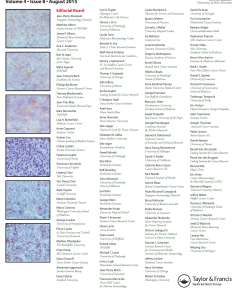
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ORIGINAL RESEARCH



Immunogenic cell death in a combined synergic gene- and immune-therapy against cancer

Benjamin Nayagam, Ikrame Amara, Meryem Habiballah, Floriane Amrouche, Philippe Beaune, and Isabelle de Waziers

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ABSTRACT

It was previously demonstrated that engineered mesenchymal stem cells (MSCs) which express a high level of a very efficient modified gene CYP2B6* (CYP2B6TM-RED) acting as a suicide gene (MSC-2B6*) in combination with cyclophosphamide (CPA) constitute a powerful cell/gene therapy approach for solid tumors. In murine models, this combination led to tumor eradication and triggered a durable immune response against tumoral cells, which prevented recurrence and metastasis.

The first goal, in this work, was to determine whether the mechanism of tumor cell death caused by CPA metabolites could explain the appearance of this anti-tumor immune response.

In vitro, CPA metabolites produced by MSC-2B6* were able to induce immunogenic cell death (ICD) of tumor cells. Indeed, all ICD characteristic events were clearly identified: calreticulin translocation, LC3II expression and release of ATP and HMGB1.

The second goal was to determine the respective roles of the direct cytotoxicity of CPA metabolites and the immune anti-tumor response due to ICD of tumor cells during tumor eradication.

In vivo, the early inhibition of ICD (with anti-HMGB1 antibodies) or the depletion of CD8⁺T lymphocytes (with anti-CD8 antibodies) prevented tumor eradication by CPA metabolites and tumor regrowth occurred, despite CPA treatment.

In conclusion, the full eradication of the tumors depends on the association of cytotoxic CPA metabolites triggering the ICD of tumor cells and an anti-tumor immune response. The absence of one or the other of these effects prevents the complete eradication of tumors.

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Introduction

An efficient method of tumor therapy consisting of sensitization of tumor cells to an anti-cancer drug by transfer of a suicide-gene has been used in our laboratory (gene-directed enzyme prodrug therapy: GDEPT). Our approach aims at introducing, specifically into tumors, a modified gene which has been constructed and patented in our laboratory (CYP2B6TM-RED = CYP2B6*).¹⁻⁴ This gene transforms very efficiently, inside the tumor, a pro-drug (cyclophosphamide, CPA) into toxic metabolites.

In immuno-competent mouse models, this suicide-gene, expressed in a tumor, allows CPA to completely eradicate the tumor, without any recurrence more than 24 months after stopping the treatment.^{3,5} In cured mice, re-challenge experiments with tumor cells, not expressing CYP2B6*, demonstrated a sustainable immune response against tumor cells mediated by cytotoxic CD8⁺T lymphocytes.^{3,5} This immune response contributes to the efficacy of this approach. It protects against recurrence of the primary tumor as well as secondary metastases.

To introduce our suicide-gene into the tumor, mesenchymal stem cells (MSCs), which migrate into the tumors and home there, were tested as vectors.⁶ MSCs expressing the suicide-gene (MSC-CYP2B6*) metabolize, inside the tumor, CPA into toxic metabolites which diffuse into neighboring tumor cells (by-stander effect). These toxic products destroy both MSC-CYP2B6* and tumor cells thus avoiding

uncontrolled proliferation and/or dissemination of transduced MSCs. Intra-tumoral administration of MSC-CYP2B6*, followed by CPA treatment, induced the complete eradication of the tumors in 30% of mice, without any recurrence/metastases more than 12 months later. The variability in the number of transduced MSCs present in the tumors after intra-tumoral injection might explain the eradication of the tumors in only one third of the mice. After re-challenge experiments with tumor cells not expressing CYP2B6*, a sustainable, anti-tumor immune reaction was observed in cured mice.⁵ This was also observed in a liver tumor rabbit model.⁷

CPA is a pro-drug which is used to treat oncogenic and autoimmune diseases.⁸ It is known that metronomic CPA treatment promotes anti-tumor immunity by selectively depleting regulatory T cells (Tregs) and, thus, by activating significant CD8⁺T cell responses.^{9,10} In our mouse models, we observed a specific decrease of Tregs upon a weekly administration of CPA.³ This Treg inhibition might explain, at least in part, the ability of our strategy to induce specific anti-tumor CD8⁺T cells.

However, it also has been shown that some anticancer therapies can lead to a particular form of cell death called “immunogenic cell death” (ICD). This death mechanism emits danger signals referred to as “damage-associated molecular patterns” (DAMPs) that induce an immune reaction, which leads to a tumor-targeted immune response and to the

establishment of immunological memory.^{11,12} The main characteristic events of ICD can be measured *in vitro* by i) the exposure of the endoplasmic reticulum chaperone calreticulin (CRT) at the surface of cells which thus enhances the uptake of tumor antigens by dendritic cells,^{13,14} ii) the increase of LC3II expression, iii) the secretion of ATP which leads to the activation of the inflammasome and the production of pro-inflammatory cytokines^{15,16} and iv) the release of the non-chromatin histone-binding protein high mobility group box 1 (HMGB1) which stimulates antigen processing and presentation to T-cells.^{17,18} Instances of cell death that fail one of these events are considered to be non-immunogenic.¹⁹⁻²¹

The first goal of this work was to determine, *in vitro*, whether the production of toxic metabolites, under the conditions of our treatment, can lead to the emission of DAMPs from tumor cells and elicit their immunogenic cell death.

The second goal was to determine, *in vivo* during the eradication phase of the tumor, the respective roles of i) the direct cytotoxicity of the CPA metabolites, ii) the ICD of tumor cells and iii) the anti-tumor immune response.

We show in this paper that this GDEPT strategy 1) leads, *in vitro*, to the emission by tumor cells of DAMPs, hallmarks of ICD; 2) prevents tumor eradication despite CPA treatment through the inhibition of ICD of tumor cells, by inhibiting HMGB1 release and 3), also prevents tumor eradication despite CPA treatment due to the depletion of CD8⁺T cells by anti-CD8 antibodies.

In conclusion, this GDEPT strategy induced, in less than two weeks, an immune response that participated in the initial eradication of tumors and was induced by the ICD of tumor cells.

Results

Identification of the molecular mechanisms of tumor cell death

Cancer cells undergoing ICD emit characteristic DAMPs.¹⁹ All these events (CRT translocation, increase of LC3II expression, ATP and HMGB1 release) were observed according to the protocol illustrated in Figure 1.

Translocation of calreticulin

The translocation of calreticulin from the endoplasmic reticulum to the cell membrane is considered as an “eat-me” signal, which stimulates cross-presentation of tumor antigens to T cells²² and promotes the uptake of cellular debris by antigen-presenting cells.^{23,24} In TC1 cells incubated for 24h with MSC either with culture medium alone or with supernatants of MSC-NI or with supernatants of MSC-CYP2B6* without any treatment, no translocation of calreticulin was observed as compared to TC1 cells grown in their culture medium (data not shown). The number of calreticulin positive TC1 cells was significantly increased ($p < .001$) when the cells were incubated with the supernatant of MSC-CYP2B6* treated with CPA as compared to TC1 cells incubated with MSC culture medium or supernatant of MSC-NI treated with CPA. Oxaliplatin (positive control) increased ($p < .05$) the number of calreticulin positive TC1 cells (Figure 2). This increase was significantly less important ($p < .001$) than that observed with the supernatant of MSC-CYP2B6* treated with CPA.

Increase of LC3II expression

Autophagy is essential for the secretion of ATP, which is a marker of ICD.²⁵ During autophagy, LC3-I is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II). The conversion of soluble LC3-I to lipid bound LC3-II is associated with the formation of autophagosomes. The increase of the LC3II/actin ratio is used as an indicator of autophagy.²⁶

TC1 cells exposed to the supernatant of MSCs expressing the CYP2B6* suicide gene and treated with CPA for 24 hours express 2.5-fold more LC3 mRNA than untreated cells as measured by RT-PCR. No difference was observed between TC1 cells exposed to the supernatant of MSC-NI treated with CPA as compared to untreated cells (Figure 3(a)). Anti-LC3B antibody detects both LC3I and LC3II proteins. By Western blotting (Figure 3(a,b)), no increase in LC3II in the tumor cells exposed to the supernatant of the MSC-NI previously treated with CPA was observed whereas a significant increase ($p < .05$) was observed in the tumor cells exposed to the supernatant of MSC-CYP2B6* treated with CPA (Figure 3(b,c)).

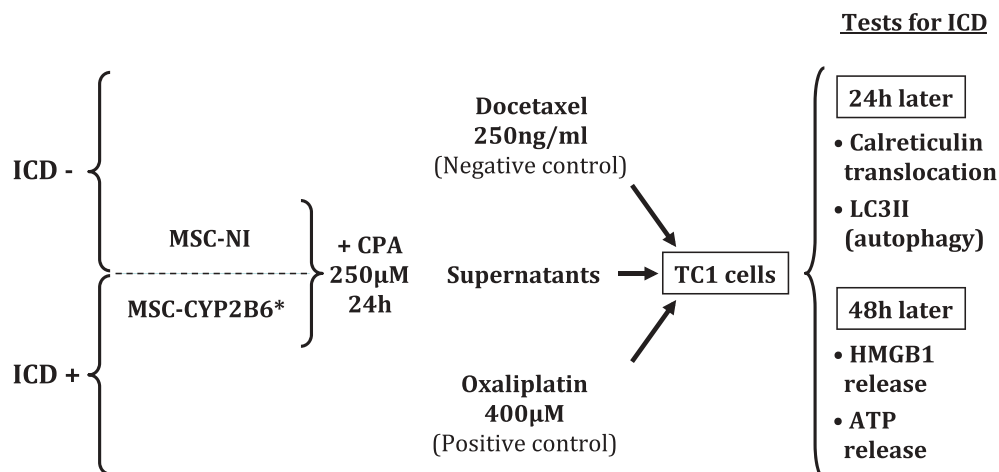


Figure 1. Protocol to detect damage-associated molecular patterns (DAMPs) specific to immunological cell death (ICD) of tumoral cells (TC1).

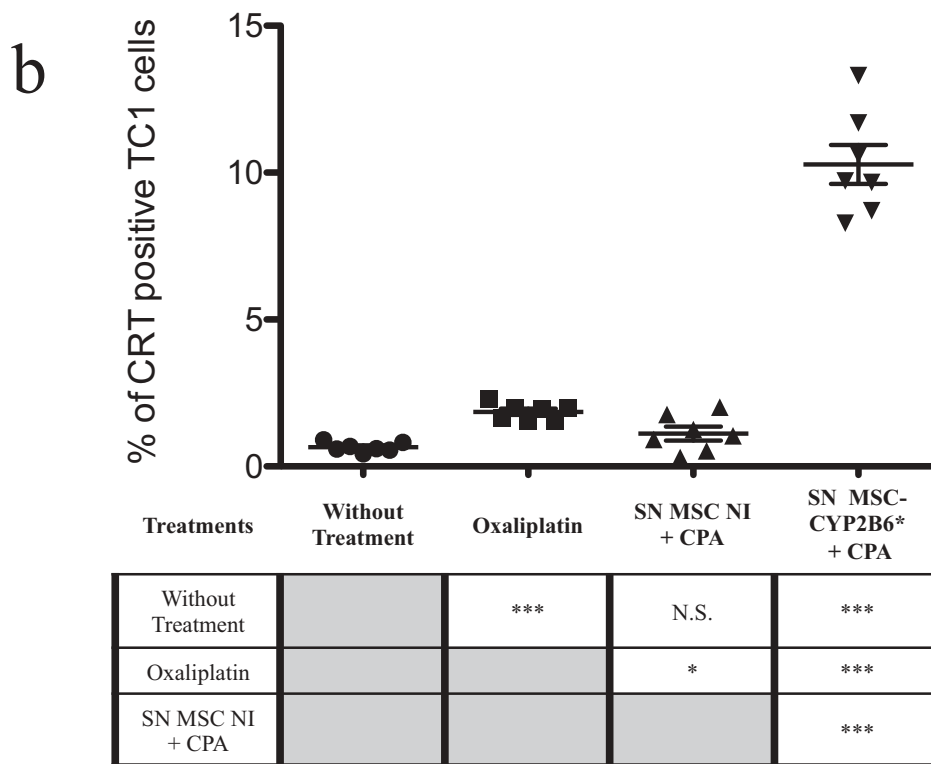
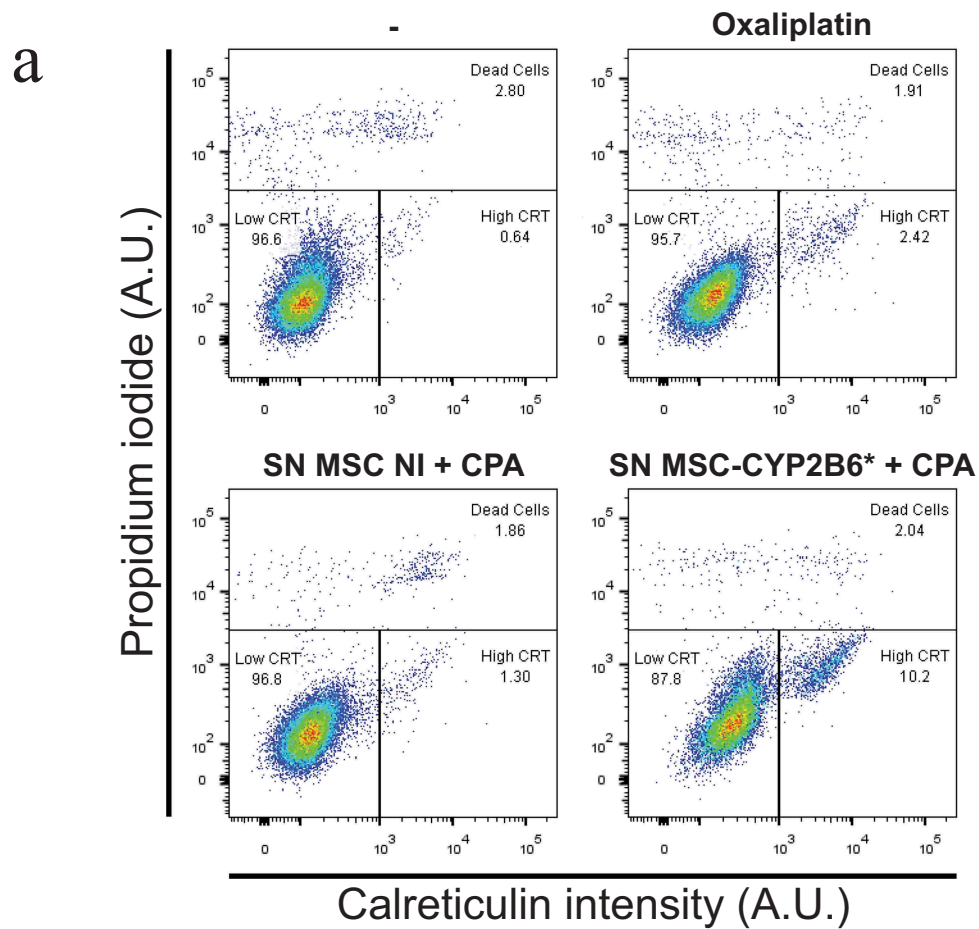


Figure 2. Calreticulin translocation in tumoral TC1 cell membranes.

TC1 cells were treated for 24h with oxaliplatin (OXA, 400 μ M) or with the supernatants (SN) of MSCs expressing or not CYP2B6* (SN MSC-CYP2B6* and SN MSC-NI, respectively) treated beforehand for 24 hours with 250 μ M of CPA. A) Dot plots of FACS analysis to detect calreticulin (CRT) positive cells and staining with propidium iodide (PI) to exclude dead cells. B) One-way analysis of variance (ANOVA) to compare experimental groups (n = 7/group) was considered significant at ***p < .001. Unpaired Student's t-tests between groups were performed: * p < .05, *** p < 0.001, NS: not significant.

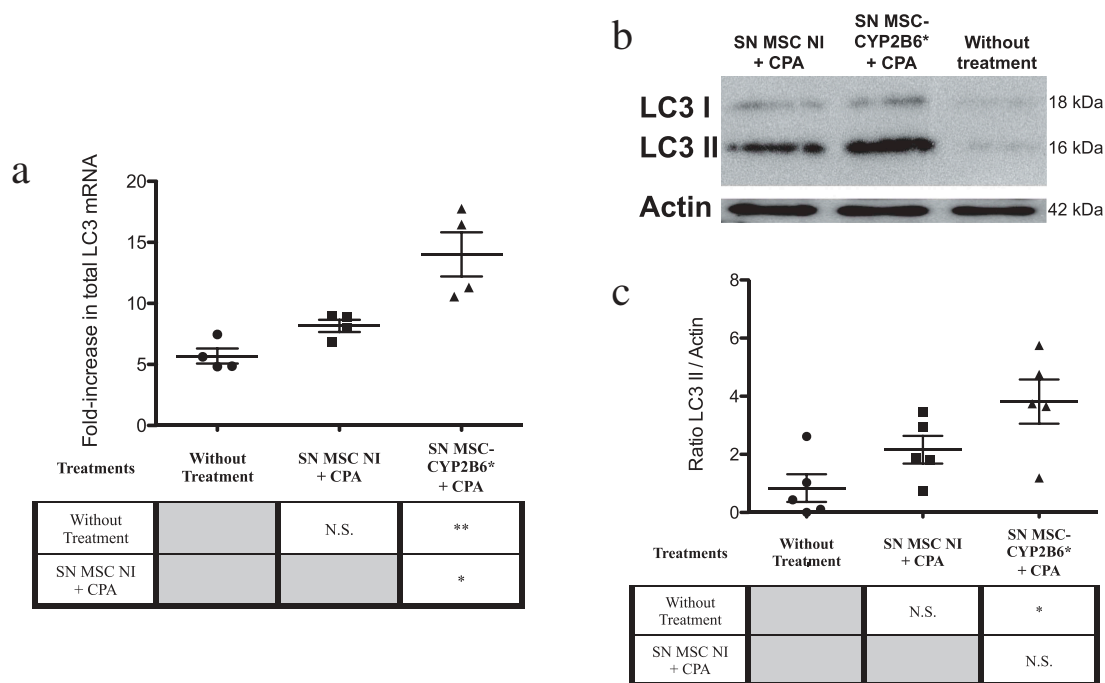


Figure 3. LC3 expression in tumoral TC1 cells.

A) Expression of LC3 mRNA in TC1 tumor cells exposed for 24 hours to supernatants of MSCs expressing or not the CYP2B6* suicide gene and treated beforehand for 24 hours with 250 μ M of CPA. The results are expressed as the fold-induction as compared to untreated cells. The values are the means for 4 independent experiments performed in triplicate. One-way analysis of variance (ANOVA) to compare experimental groups was considered significant at ** $p < .01$. Unpaired Student's t-tests between groups were performed: * $p < .05$, ** $p < .01$; B) Tumor cell homogenates (20 μ g) exposed for 24 hours to the supernatants of MSCs expressing or not the CYP2B6* suicide gene and treated beforehand for 24 hours with 250 μ M of CPA were analyzed by western blotting with anti-LC3 antibody; C) LC3II protein levels were quantified using actin as an internal standard and the results are presented as the ratio LC3II/actin. The values are the means of 4–5 independent experiments performed in triplicate; One-way analysis of variance (ANOVA) to compare experimental groups was considered significant at ** $p < .01$. Unpaired Student's t-tests between groups were performed: * $p < .05$, NS: not significant.

ATP assays

Quinacrine is a fluorescent dye that has been reported to specifically stain ATP-containing vesicles.²⁷ It, thus, allows the study of variations in intracellular ATP under different conditions. The reduction of quinacrine-dependent fluorescence occurred in cells that had an intact plasma membrane. In Figure 4(a), two distinct cell populations are apparent in the dot plot derived from untreated cells (control). One minor population is constituted by dead cells, which stain with propidium iodide (PI) equal or superior to 3×10^3 A.U. The largest population (95%) represents living cells which contain high levels of ATP (from 10^3 to 10^4 A.U. quinacrine) and less than 3×10^3 A.U. of PI staining. Dot plots from TC1 cells exposed to docetaxel or to the supernatant of MSC-NI previously treated with CPA are similar to those of control cells (data not shown). After treatment with oxaliplatin, an ICD inducer, a new cell population appears called here “dying cells”. They are characterized by weak PI staining ($< 3 \times 10^3$ A.U., cells not dead) and a low intracellular ATP content (between 50 to 150 A.U. quinacrine). Dot plots from TC1 cells exposed to the supernatant of MSCs expressing CYP2B6* and previously treated with CPA are comparable to those of TC1 cells treated with oxaliplatin. They also exhibit the emergence of a “dying cells” population. The percentage of “dying cells” was measured under several conditions (Figure 4(b)). It is around 9% when TC1 cells were treated with oxaliplatin (400 μ M) or with the supernatant of MSC-CYP2B6* previously treated with CPA whereas in all other conditions this percentage is inferior to 1%. This indicates that this

population of “dying cells” is increased by these two treatments (oxaliplatin or the supernatant of MSC-CYP2B6*).

Further, triple staining was performed to measure calreticulin translocation in living and “dying” cells (Figure 4(c)). In live cells, calreticulin expression was detected at the same level (about 130 A.U.) in untreated cells and in cells treated with oxaliplatin or with the supernatant of MSC-NI previously treated with CPA. In contrast, calreticulin expression was significantly increased ($p < .001$) in living cells after docetaxel treatment or after incubation with the supernatant of MSC-CYP2B6* previously treated with CPA (about 230 A.U.). In “dying cells”, calreticulin expression was much higher after oxaliplatin treatment as compared living cells (about 610 A.U.) and it was even higher when cells were exposed to supernatant of MSC-CYP2B6* previously treated with CPA (about 950 A.U.).

The percentage of “dying TC1 cells” increased with increasing ratios (0.5 to 6) of MSC-CYP2B6* treated with CPA to TC1 cells (Figure 5(a,b)). In comparison, increasing the ratios of MSC-NI treated with CPA to TC1 cells did not increase the percentage of “dying cells” (Figure 5(b)). A positive correlation also was observed between the percentage of “dying cells” and the dose of oxaliplatin (Figure 5(c)) whereas increasing doses of docetaxel did not cause the appearance of “dying cells” (Figure 5(d)).

HMGB1 release

HMGB1 is the most abundant non-histone, nuclear protein. It acts as a DNA-binding protein in the nucleus to sustain chromatin

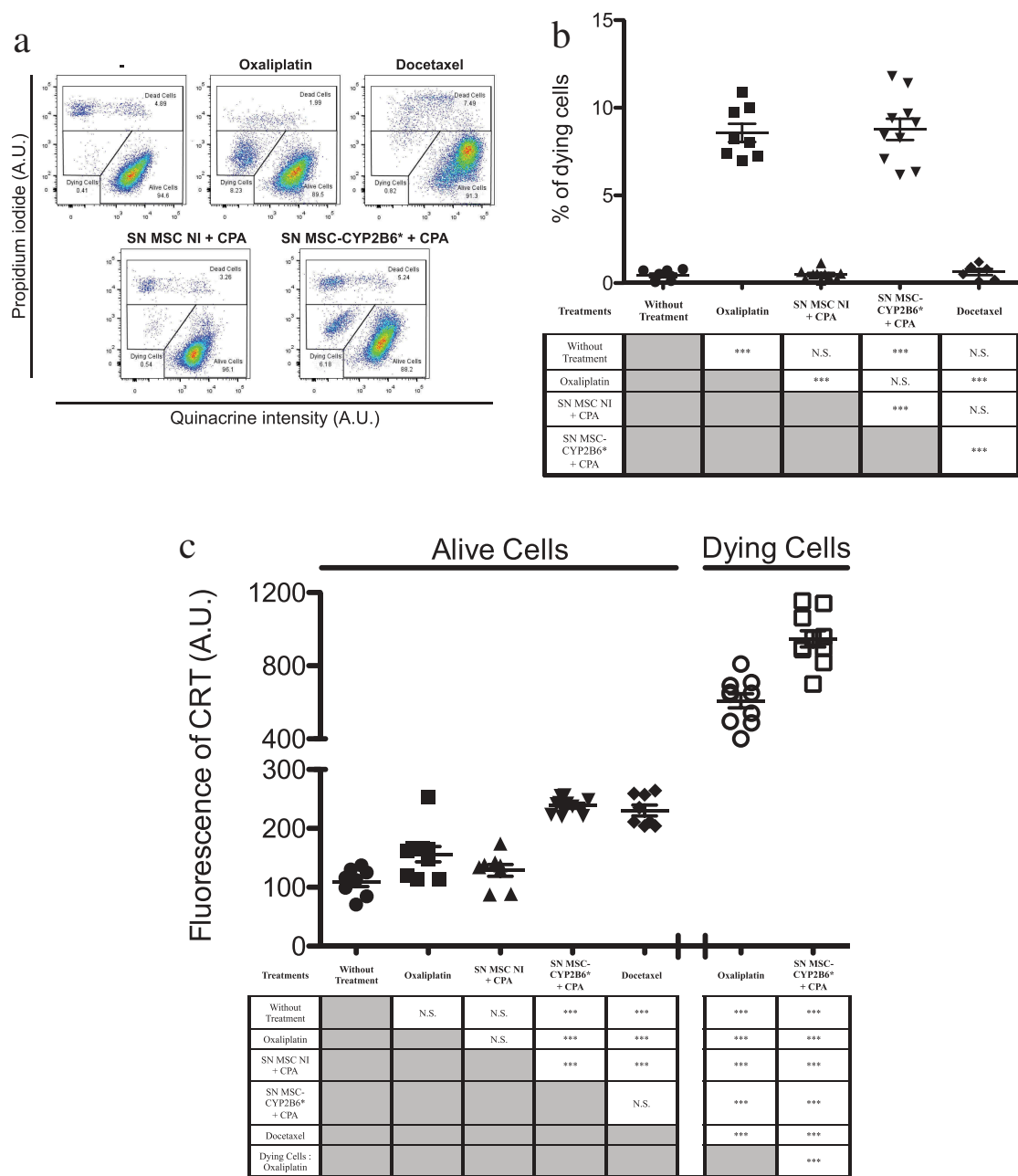


Figure 4. ATP and CRT expression in tumoral TC1 cells.

TC1 tumor cells exposed for 48 hours to oxaliplatin (OXA, 400 μ M), docetaxel (250 ng/mL) or supernatants of MSCs expressing or not the CYP2B6* suicide gene and treated beforehand for 24 hours with 250 μ M of CPA were analyzed by FACS. A) Dot plots for the different conditions used in the FACS analysis after the staining of ATP and of PI, to exclude dead cells from the analysis. Three cell populations were characterized; living cells, dead cells, and “dying cells”. B) Percentage of “dying cells” in the experimental groups. The results are expressed as the means \pm SEM (n = 8-10/group). One-way analysis of variance (ANOVA) to compare experimental groups was considered significant at *** $p < .001$. Unpaired Student’s t-tests between groups were performed: *** $p < 0.001$, NS: not significant. C) After staining for ATP and PI, fluorescence intensity of CRT was measured in living cells and “dying cells”. The results are expressed as the means \pm SEM (n = 8-10/group). One-way analysis of variance (ANOVA) to compare experimental groups was considered significant at *** $p < .001$. Unpaired Student’s t-tests between groups were performed: *** $p < 0.001$, NS: not significant.

structure and it regulates DNA repair. The function of HMGB1 depends upon its localization.²⁸ In the extracellular medium, HMGB1 operates as a potent pro-inflammatory stimulus.²⁹ HMGB1 release was observed when TC1 cells were treated with oxaliplatin (positive control for immunogenic cell death³⁰) and this release was more important when cells were treated with the supernatant of MSC-CYP2B6* treated with CPA. No HMGB1 release was observed in untreated TC1 cells or in TC1 cells

incubated with docetaxel or with the supernatant of MSC-NI treated with CPA (Figure 6).

Evaluation in vivo of the role of ICD in the eradication of the tumor

The monoclonal anti-HMGB1 antibodies used in this study have been characterized extensively with respect to their HMGB1

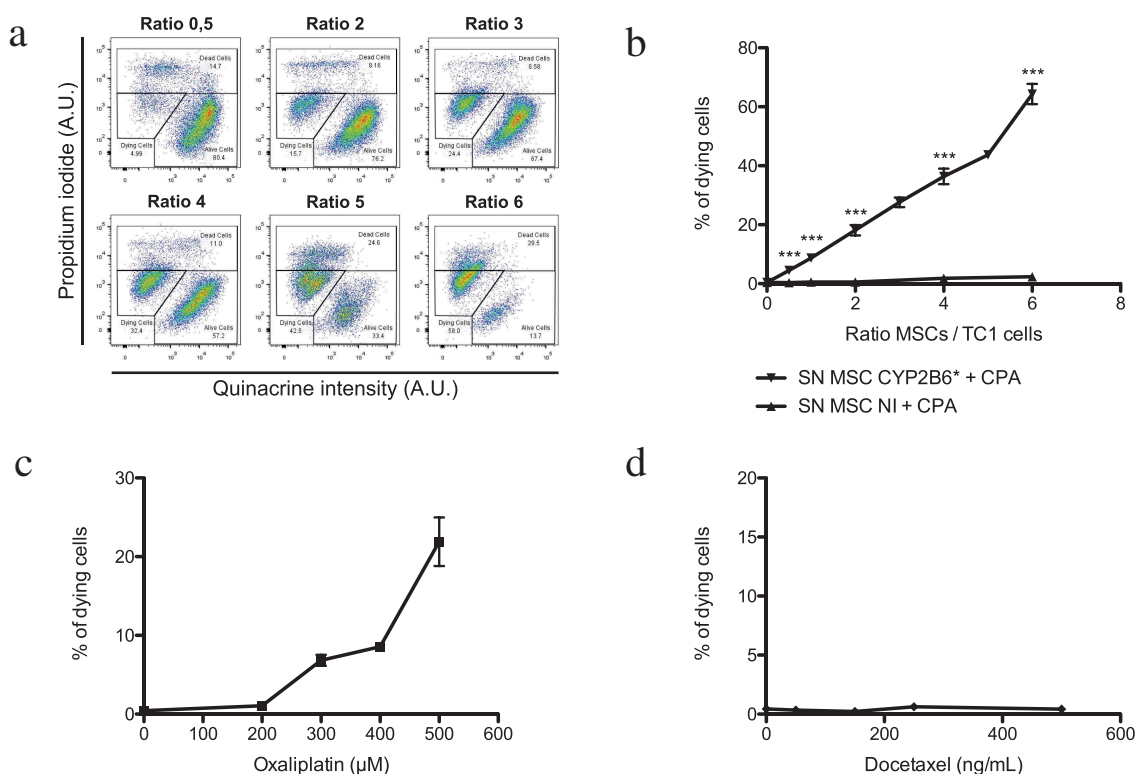


Figure 5. Kinetic analysis of "dying cells".

A) Different amounts of MSC-CYP2B6* (from 0.5×10^5 up to 6×10^5 cells) were treated for 24 hours with $250 \mu\text{M}$ of CPA, then the supernatants (SN) were incubated with 10^5 TC1 cells for 48 hours (MSCs/TC1 cell ratios ranging from 0.5 to 6). Dot plots for the different conditions used in FACS analysis after the staining of ATP and PI, to exclude dead cells from the analysis. B) Different amounts of MSC-NI or MSC-CYP2B6* (from 0.5×10^5 up to 6×10^5 cells) were treated for 24 hours with $250 \mu\text{M}$ of CPA, then the supernatants (SN) were incubated with 10^5 TC1 cells for 48 hours (MSCs/TC1 cell ratios ranging from 0.5 to 6). The percentage of "dying cells" was determined in each condition. Statistical analysis for each ratio according to the supernatant used was performed with unpaired Student's t-tests *** $p < .001$. C) Percentage of "dying cells" in TC1 treated with oxaliplatin (0-500 μM) and D) Percentage of "dying cells" in TC1 treated with docetaxel (0-500ng/ml). After staining of ATP, the percentages of "dying cells" are expressed as the means \pm SEM (n = 3-9/condition).

neutralizing activity in *in vitro* and *in vivo* studies.^{31,32} In order to evaluate, *in vivo*, the role of ICD of tumor cells in tumor eradication, we compared the effects of CPA in tumors induced with TC1-CYP2B6*, in immuno-competent mice, by blocking, or not, ICD of tumor cells with anti-HMGB1 antibodies (Figure 7). Without CPA treatment, tumors grew exponentially and anti-HMGB1 administration was without effect on tumor growth. Four injections of CPA, in mice bearing TC1-CYP2B6* tumors, resulted in complete eradication of the tumor whereas anti-HMGB1 injections prevented this eradication and tumor growth resumed at the end of treatments by CPA and anti-HMGB1 antibodies.

Evaluation *in vivo* of the roles of CPA metabolite-related toxicity and the immune response against tumor cells in the eradication of the tumor

Our previous results demonstrated that the immune response protecting against relapse, in our GDEPT strategy, was mediated by CD8⁺T cells.^{3,5} In order to evaluate the role of the immune response in the initial eradication of the tumors, we performed *in vivo* studies using tumors induced in immune-competent mice with TC1 or TC1-CYP2B6* cells. In both cases, we compared the effects of CPA in the presence

or absence of an immune response (blocked by the administration of anti-CD8 antibodies).

Following subcutaneous injections of TC1 or TC1-CYP2B6* cells, tumors grew exponentially at similar rates in the absence of CPA, as previously observed.³ Weekly injections of anti-CD8 antibodies, when the tumor reached 500 mm³ until the sacrifice of the mice, did not modify the tumor growth (Figure 8(a,b)).

In mice bearing TC1 tumors, four weekly injections of 90mg/Kg CPA, the dose used in our previous work,⁵ slowed tumor growth which resumed at the end of the treatment, as previously observed.^{3,5} Prior injection of anti-CD8 antibodies limited the effect of CPA and mice were sacrificed sooner (Figure 8(a)). In mice bearing TC1-CYP2B6* tumors, the tumor volume decreased at the first CPA injection and 4 injections of CPA resulted in complete eradication of the tumor. The depletion of CD8⁺T cells with anti-CD8 antibodies prevented this eradication and tumor growth resumption was significant ($p < .05$) around 10 days after the second injection of CPA (Figure 8(b)).

Discussion

It has been observed that, beyond their cytotoxic properties, numerous anticancer drugs can stimulate the immune system and, in some instances, even produce long-term protective

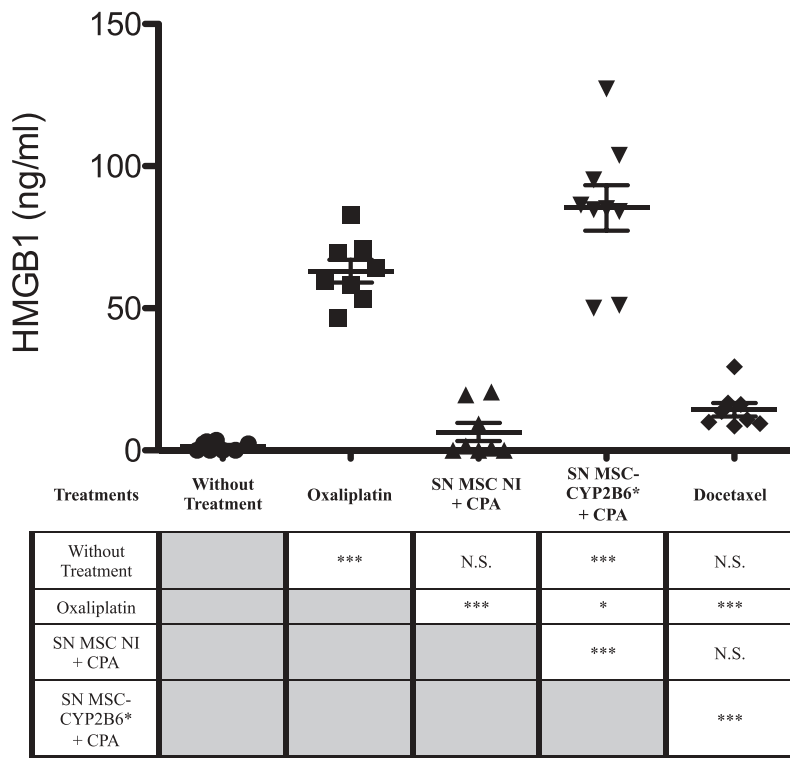


Figure 6. HMGB1 release by tumoral TC1 cells.

TC1 cells were treated for 48 h with oxaliplatin (400 μM), docetaxel (250 ng/mL) or with the supernatants (SN) of MSC-NI and MSC-CYP2B6* treated beforehand for 24 hours with 250μM of CPA. HMGB1 release was detected in the supernatants with the Elisa kit from IBL International. One-way analysis of variance (ANOVA) to compare experimental groups (n = 7-9/group) was considered significant at *** *p* < .001. Unpaired Student’s t-tests between groups were performed: * *p* < .05, *** *p* < .001, NS: not significant.

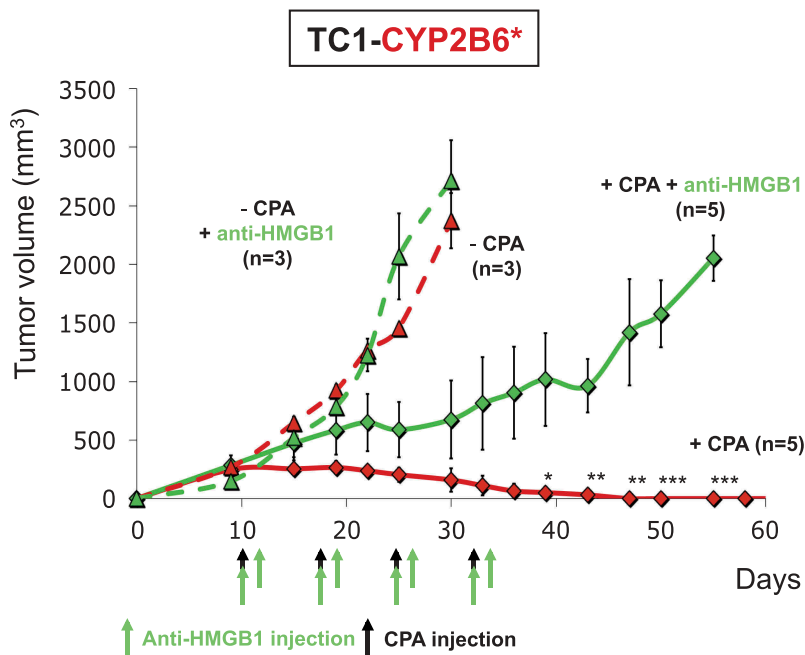


Figure 7. Effects of HMGB1 depletion on mice with tumors resulting from TC1-CYP2B6* cells treated or not with CPA.

2x10⁵ TC1-CYP2B6* were injected, subcutaneously, into the right flanks of mice. When the tumors reached about 250 mm³, mice received (full lines) or not (dotted lines) injections of 90 mg/Kg CPA (4 injections, i.p. once a week). To maintain HMGB1 depletion (green lines), intra-peritoneal injections of anti-HMGB1 antibodies (50 μg/mouse) were performed twice a week, the first one just after the injection of CPA, and the second one two days later. Tumor volumes were measured using a Vernier caliper twice a week and expressed as the volume (mm³) = length x width²/2. The results are expressed as the means ± SEM and statistical analysis among groups (with or without anti-HMGB1 antibodies) was performed by unpaired Student’s t-tests * *p* < .05, ** *p* < .01, *** *p* < .001.

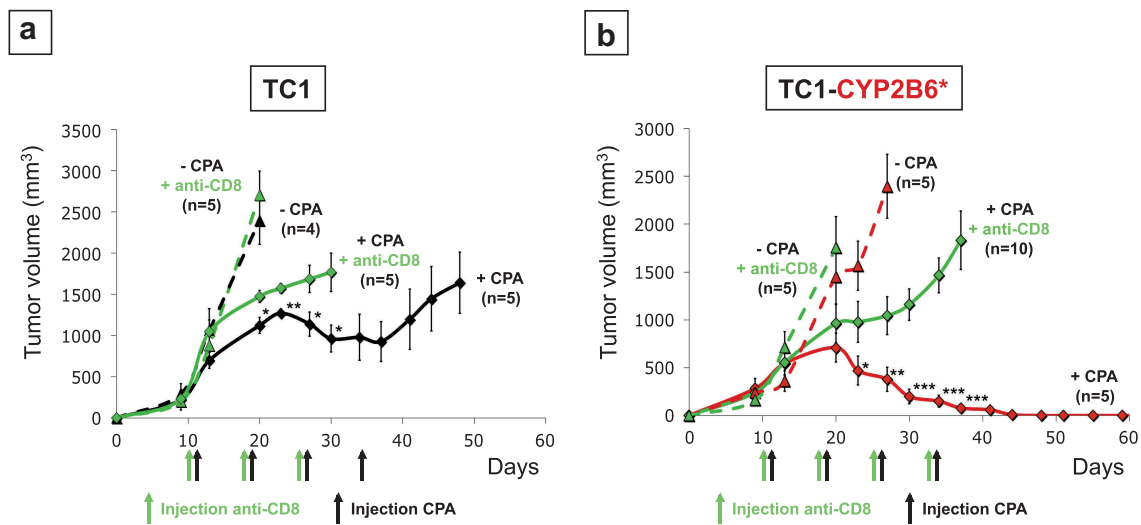


Figure 8. Effects of CD8 depletion on mice with TC1 (A) or TC1-CYP2B6* (B) cell-induced tumors treated or not with CPA.

5x10⁵ TC1 (black lines) or TC1-CYP2B6* (red lines) were injected, subcutaneously, into the left flanks of mice. When the tumors reached about 500 mm³, mice received (full lines) or not (dotted lines) injections of 90 mg/Kg CPA (4 injections, i.p. once a week). To maintain CD8 depletion (green lines), intra-peritoneal injections of anti-CD8 antibodies (100µg/mouse) were performed the day before the injection of CPA and repeated once a week until the sacrifice of the mice. Tumor volumes were measured using a Vernier caliper twice a week and expressed as the volume (mm³) = length x width²/2. The results are expressed as the means ± SEM and statistical analysis among groups (with or without anti-CD8 antibodies) was performed by unpaired Student's t-tests * $p < .05$, ** $p < .01$, *** $p < .001$.

memory T cell responses which are necessary for the effectiveness of chemotherapy leading to the tumor eradication.³³

In mouse models,^{3,5} we have shown previously that CPA eradicated ectopic pulmonary tumors (TC1-CYP2B6*) without any recurrence for up to 24 months after stopping the treatment. Moreover, in cured mice, re-challenge with TC1 tumor cells without CYP2B6* led to the formation of small tumors that regressed spontaneously 7 days after cell inoculation without any treatment. This immune response was, therefore, directed against TC1 cells and not against CYP2B6* and was mediated by anti-CD8⁺T cells^{3,5} which protected against a recurrence and the appearance of metastases. This effect also was observed in a rabbit VX2 tumor hepatic model.⁷ However, the respective roles of the cytotoxicity of the CPA metabolites and the immune response in the initial tumor eradication remained unknown.

It can be hypothesized that CPA acts on the immune response at two levels: i) it depletes Tregs^{3,9,10} and assists in the development of the antitumor immune response and ii) cytotoxic CPA metabolites induce immunogenic cell death of tumor cells which stimulates a specific anti-tumor immune response.

Tregs are involved in tumor progression^{34,35} and contribute to immune tolerance to cancer.³⁶ In previous work, we showed that the administration of CPA selectively depleted Tregs in murine tumor models.³ Ghiringhelli *et al.* demonstrated that such a CPA regimen also decreased Tregs in humans while preserving other lymphocyte subsets in both number and function.^{37,38} Therapeutic depletion of these cells is known to improve responses to cancer immunotherapy³⁹ since it can induce the production of type I interferons which boost the differentiation and mobilization of mature dendritic cells (DCs) and expand T cells with a memory phenotype.⁴⁰ However, the depletion of Tregs only is not sufficient to reduce tumor growth (Figure 8(a,b)).

The complete tumor eradication that was observed was due, in part, to the immune response. In order to more fully explain

the eradication, it was hypothesized that cytotoxic metabolites of CPA can trigger an immunogenic cell death (ICD) of tumoral cells. Indeed, all four signals associated with this ICD, i) calreticulin translocation, 2) LC3II expression, 3) ATP release and 4) HMGB1 release,¹⁹ were detected in this work.

When added to tumor cells (TC1), the supernatant of MSC-CYP2B6* incubated for 24h with CPA induced calreticulin translocation (Figure 2) and HMGB1 release (Figure 6) after 24h or 48h, respectively. The amounts of these DAMPs were significantly higher as compared to TC1 cells treated with oxaliplatin, a positive control for ICD.²¹ Both calreticulin translocation and HMGB1 release also were observed when mafosfamide, which non-enzymatically produces 4-OH-CPA, was incubated with EG7 lymphoma cells.²² On the other hand, these two DAMPs were undetectable when TC1 cells were treated with our negative controls: docetaxel and the supernatant of MSC-NI treated with CPA (Figures 2 and 6).

Moreover, the autophagy essential for ICD induced the secretion of ATP²⁵ as shown by the increase of the LC3II/actin ratio (Figure 3). Quinacrine is a fluorescent dye that stains intracellular ATP.²⁷ A partial reduction of quinacrine-dependent fluorescence was observed after staining of human osteosarcoma cells (U2OS) treated with mitoxantrone, a classical inducer of immunogenic cell death.⁴¹ The reduction of quinacrine-dependent fluorescence occurred in TC1 cells exposed for 48h to oxaliplatin or to the supernatant of MSC-CYP2B6* treated beforehand for 24h with CPA. This reduction was observed in cells which have an intact plasma membrane as determined with the vital dye PI (Figure 4(a,b)). They are the so called “dying cells” since they are still alive, but they have released their ATP, their main source of energy. These “dying cells” seem to be a specific marker of immunogenic death since they were not detectable after treatment with our two negative controls (docetaxel and supernatant of MSC-NI treated beforehand with CPA). Furthermore, they seem to play a key role in ICD since their percentage was

correlated both to the dose of oxaliplatin and to the dose of cytotoxic metabolites produced by an increasing number of transduced MSCs exposed for 24 hours to the same dose of CPA (Figure 5). In comparison, with increasing amounts of MSC-NI treated beforehand with CPA or with increasing doses of docetaxel, the size of this population of “dying cells” remained extremely small (figure 5(b,d)). Finally, these “dying cells” had a very marked translocation of CRT as compared to living cells (Figure 4(c)) which confirmed that the “dying cells” moved calreticulin to the membrane and released their ATP, both signals of ICD.

ICD is characterized by specific signals that we have identified clearly *in vitro*. It is known that the blockage of one of these signals prevents the immunogenic death of tumor cells.^{19–21} To verify that the immunogenic death of tumor cells plays an essential role in the eradication of tumors, we developed an *in vivo* mouse model by inhibiting HMGB1 with anti-HMGB1 antibodies. We demonstrated (Figure 7) in mice bearing TCI-CYP2B6* tumors that CPA cannot eradicate tumors in absence of ICD of tumor cells.

A T cell response typically peaks ~7–15 days after initial antigen stimulation.⁴² In our study, blocking the immune response by the administration of anti-CD8 antibodies began to prevent the tumor eradication roughly 2 weeks after the beginning of the treatment (Figure 8). The immune response against tumor cells appears in a second phase and is mandatory for the efficiency of our strategy. Thus, it is clear that the full eradication of tumors needs the association of cytotoxic CPA metabolites which lead both to ICD of tumor cells and then to an immune response since the absence of one or the other prevents this eradication.

Critical conditions to define a good inducer of ICD have been defined^{43–45} and our approach has been shown to fulfill these conditions:

- (1) The treatment needs to induce a large emission of multiple DAMPs. This work has demonstrated that our strategy triggered all these major danger signals sometimes with responses greater than with oxaliplatin, a known inducer of ICD.
- (2) The cytotoxic metabolites have to stay in the cells long enough to induce ICD. Our approach demonstrated that CPA cytotoxic metabolites, produced by the transduced MSCs, diffused into the culture medium, penetrated into tumor cells and killed them through ICD (by-stander effect).
- (3) An ICD inducer should cause severe endoplasmic reticulum (ER) stress. Figure 2 shows that our strategy promoted a major ER stress which led to a translocation of CRT that was greater than that produced by oxaliplatin, a paradigmatic ICD inducer which was used as a positive control.
- (4) Strong immunogenicity of the DAMPs is required to mediate an antitumor immunity. Our approach triggered an immune protection as demonstrated by re-challenge experiments. It played a role in the primary tumor eradication^{3,5} (Figure 6, 7).
- (5) The drug should not have inhibitory or suppressive effects on the immune system. It is known that

metronomic administration of CPA selectively inhibits T_{reg} cells while preserving other lymphocyte subsets in both number and function,^{3,37,38}

- (6) The ICD inducer needs to lead not only to the eradication of the tumor but also to metastasis. We demonstrated that trans-arterial administration of transduced MSCs and CPA injection resulted in 78% tumor necrosis and less metastasis in a rabbit VX2 hepatic tumor model.⁷

In conclusion, both *in vitro* and *in vivo* experiments present ample evidence that cytotoxic CPA metabolites induce a strong tumoral ICD that triggers an adaptive and specific anti-tumoral immune response that is essential for tumor eradication

Materials and methods

Cell cultures

TC1 cells, derived from a murine lung epithelial-cell line, which express firefly luciferase,⁴⁶ were cultured as previously described.³ Murine mesenchymal stem cells (MSCs) from the bone-marrow of C57BL/6 mice were provided by Life technologies and were cultured in DMEM High glucose medium with L-glutamine, without sodium pyruvate or HEPES (GIBCO), supplemented with 10% fetal calf serum, streptomycin (50 µg/mL), penicillin (200 UI/mL), fungizone (0.5 µg/mL) and sodium pyruvate (1 mM). MSCs were transduced with lentiviral particles expressing our suicide gene (LV-CYP2B6TM-RED = LV-CYP2B6*). The clone 42 (MSC-CYP2B6*), which expresses the highest level of transgene catalytic expression, was selected as previously described⁵ and used for all the following experiments.

Damps detection

Translocation of calreticulin

10⁵ TC1 cells were treated for 24 hours with or without oxaliplatin (400 µM – Sigma, Ref: O9512) and used, respectively, as positive and negative controls. 10⁵ non-infected MSCs (MSC-NI) and MSC-CYP2B6* were treated with 0.25 mM of CPA for 24 hours. Supernatants (SN) were incubated with 10⁵ TC1 cells for 24 hours. Cells were harvested, washed in PBS and nonspecific receptors were blocked with 5% normal goat serum in PBS for 20 min at 37°C. Then, cells were washed in PBS and stained with anti-mouse calreticulin antibody (1 µg/10⁶ cells) (Abcam, ab22683) for 20 min at room temperature, then with Alexa-Fluor 647 goat anti-mouse IgG at 1/400 (Life Technologies, A21240) for 20 min at room temperature. To evaluate cell viability and exclude dead cells, they were incubated with 1 µg/mL of propidium iodide (PI) and then analyzed by FACS.

HMGB1 release

TC1 cells, treated or not with oxaliplatin (400 µM) or docetaxel (250 ng/mL), diluted in MSC culture medium for 48h were used as positive and negative controls, respectively. 10⁵ MSC-NI and MSC-CYP2B6* were treated with 0.25 mM of CPA for 24 hours, then the supernatants (SN) were incubated

with 10^5 TC1 cells for 48 hours. HMGB1 release was detected in the supernatants with the Elisa kit from IBL International according to the manufacturer's instructions.

Autophagy

2.5×10^5 non infected MSCs (MSC-NI) and MSC-CYP2B6* were treated with 0.25 mM of CPA for 24 hours, then the supernatants (SN) were incubated with 2.5×10^5 TC1 cells for 24 hours.

- (1) Total RNA from TC1 cells was isolated using RNeasy Mini kit (Qiagen) and then reverse-transcribed using High Capacity cDNA archive kit (Applied Biosystems). LC3 mRNA content was determined using the following primers Up 5' CATGCCGTCCGAGAAGACCT 3' and low 5' GATGAGCCGGACATCTTCCACT 3' and was normalized on the basis of TATA-binding protein (TBP) content using as primers Up 5' TGCACAGGAGCCAAGAGTGAA 3' and low 5' CACTCACAGCTCCACCA 3' using the $2^{-\Delta\Delta C_t}$ method. PCR reactions were performed using an ABI prism 7700 sequence detection system (Applied Biosystems) and the SYBR Green PCR Core Reagents kit (Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 40 cycles at 95°C for 15 s and 65°C for 1 min.
- (2) Cells were trypsinized, centrifuged and the pellet was incubated in RIPA buffer (Thermo Fischer Scientific) containing 15% of complete protease inhibitor cocktail (Roche) for 10 min at 4°C. After centrifugation at 12,500g at 4°C for 10 min, protein concentrations were determined using the bicinchoninic acid (BCA) procedure according to the manufacturer's instructions (Pierce). 20 µg of total protein were loaded onto 12% SDS/polyacrylamide gels. The proteins were transferred onto polyvinylidene difluoride membranes (PVDF) (Hybond P, GE Healthcare Europe) and probed with polyclonal rabbit anti-LC3B purchased from Cell signaling (ref 2775S, Ozyme). The membranes were washed, exposed to a secondary antibody anti-rabbit IgG linked to HRP and developed using an ECL kit (Bio-Rad). To ensure the quantity of loaded proteins, actin protein has been quantified. Images were then analyzed using ImageJ software.

ATP assays

10^5 non-infected MSCs (MSC-NI) and MSC-CYP2B6* (clone 42) were treated with 0.25 mM of CPA for 24 hours. Then, the supernatants (SN) of culture cells were incubated with 10^5 TC1 cells for 48 hours. Cells treated for 48h with oxaliplatin (400 µM – Sigma) were used as a positive control¹¹ and cells treated with docetaxel (250 ng/mL – Sigma) for 48h were used as a negative control.⁴⁷ For the detection of ATP containing vesicles, the cells were labeled with quinacrine.²³ Briefly, cells were harvested, washed in PBS and labeled with 1 µM quinacrine in Krebs-Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 0.7 mM KH₂PO₄, 2 mM CaCl₂, 6 mM glucose and 25 mM HEPES, pH 7.4), then washed and resuspended in PBS containing 1 µg/mL of propidium iodide (PI). The effect

of varying the MSCs/TC1 cell ratio was also evaluated. Different amounts of MSCs, from 0.5×10^5 up to 6×10^5 cells were treated with 0.25mM of CPA for 24 hours, then the supernatants (SN) were incubated with 10^5 TC1 cells for 48 hours, namely MSCs/TC1 cell ratios ranging from 0.5 to 6. The effects of increasing amounts of oxaliplatin (0-500µM) and docetaxel (0-500ng/ml) also were studied.

Fluorescence activated cell sorting (FACS)

Flow cytometry analysis was performed with a BD Canto II using flow cytometry Diva software (BD Biosciences). FlowJo software (Treestar) was used to analyze data.

Animal models

In vivo studies were performed on 11-week-old female C57Bl/6Jrj mice (Janvier). All animal experiments were conducted in accordance with ethical guidelines and were approved by the animal ethics committee of the Paris Descartes University (CEEA34.IW.03#.11, CEEA.IDW.097.12). Tumor growth was evaluated twice weekly by Vernier caliper measurement and was expressed as the volume (mm^3) = $(L \times l^2)/2$.

Depletion of HMGB1

2×10^5 TC1-CYP2B6*, were diluted in 200 µL PBS and injected into the subcutaneous tissue of the flanks of mice. When the tumor size approach 250 mm^3 , mice bearing TC1-CYP2B6* tumors were divided into four groups: i) one group received no treatment, ii) one group was treated intraperitoneally with monoclonal anti-HMGB1 antibodies (clone 2G7, IgG2b, provided by Dr. Kevin Tracey of the Feinstein Institute for Medical Research) (50 µg/mouse) twice a week until the sacrifice of the mice, iii) one group was treated with CPA (Endoxan, Baxter) diluted in sterile PBS, at a dose of 90 mg/Kg, intraperitoneally, once a week for 4 weeks and iv) one group was treated with CPA similarly to the third group and, in addition, intra-peritoneal injections of anti-HMGB1 antibodies (50 µg/mouse) were performed twice a week, the first one just after the injection of CPA, and the second one two days later.³¹ Mice were sacrificed when the tumor volume reached 2 000 mm^3 .

Depletion of CD8 ± T cells

5×10^5 TC1 cells, with or without previous LV-CYP2B6* infection, were diluted in 50 µL PBS and injected into the subcutaneous tissue of the flanks of mice, after anesthesia, as previously described.³ When the tumor size approach 500 mm^3 , mice bearing either TC1 or TC1-CYP2B6* tumors were divided into four groups: i) one group received no treatment, ii) one group was treated intraperitoneally with anti-CD8 antibodies (100 µg/mouse, Euromedex) once a week until the sacrifice of the mice, iii) one group was treated with CPA (Endoxan, Baxter) diluted in sterile PBS, at a dose of 90 mg/Kg, intraperitoneally, once a week for 4 weeks and iv) one group was treated with CPA similarly to the third group but, in addition, the day before each CPA treatment they were injected, intraperitoneally, with anti-CD8 antibodies (100µg/mouse, Euromedex). To maintain depletion of CD8, anti-CD8 antibody injections were

repeated once a week until the sacrifice of the mice. Mice were sacrificed when the tumor volume reached about 1700–2000 mm³.

Statistical analysis

Data are presented as the means ± SEM. One-way analysis of variance (ANOVA) was used to compare the means from multiple experimental groups, followed by two-tailed Student's t-test to compare groups between one another. Statistical differences were considered to be significant when $p < .05^*$, $p < .01^{**}$ or $p < .001^{***}$.

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Disclosure of potential conflicts of interest

Isabelle de Waziers and Philippe Beaune are co-founders of the COGITH company.

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