Oncogene (2015), 1–8

www.nature.com/onc

ORIGINAL ARTICLE

Hyperthermic intraperitoneal chemotherapy leads to an anticancer immune response via exposure of cell surface heat shock protein 90

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The occurrence of peritoneal carcinomatosis is a major cause of treatment failure in colorectal cancer and is considered incurable. However, new therapeutic approaches have been proposed, including cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC). Although HIPEC has been effective in selected patients, it is not known how HIPEC prolongs a patient's lifespan. Here, we have demonstrated that HIPEC-treated tumor cells induce the activation of tumor-specific T cells and lead to vaccination against tumor cells in mice. We have established that this effect results from the HIPEC-mediated exposure of heat shock protein (HSP) 90 at the plasma membrane. Inhibition or blocking of HSP90, but not HSP70, prevented the HIPEC-mediated antitumoral vaccination. Our work raises the possibility that the HIPEC procedure not only kills tumor cells but also induces an efficient anticancer immune response, therefore opening new opportunities for cancer treatment.

Oncogene advance online publication, 13 April 2015; doi:10.1038/onc.2015.82

INTRODUCTION

Peritoneal carcinomatosis (PC) is a major cause of treatment failure in the management of several abdominal cancers, including colorectal and ovarian cancers. Indeed, PC has long been considered a terminal condition and patients are generally given palliative treatment. Patients with limited PC that receive standard treatment (surgery and systemic chemotherapy) have a median survival of 24 months.[1] However, the treatment strategies have changed over the last few decades and options for prolonged survival are now available. The finding that some patients with PC have a regional disease rather than a systemic malignancy has led to the development of a novel strategy that combines cytoreductive surgery of all macroscopic intraperitoneal tumors with hyperthermic intraperitoneal chemotherapy (HIPEC). This combined treatment is increasingly accepted as the best therapeutic option for some types of peritoneal malignancies.[2] In brief, the current HIPEC procedure is as follows: the PC index is evaluated to determine whether the HIPEC treatment is suitable for the patient (on average, 40–50% of patients are suitable). After resection/cytoreduction of all the affected tissue (for example, the peritoneum, spleen, digestive track), HIPEC can be initiated. In this procedure, the chemotherapy solution is warmed to 42 °C and administered per operatory for the microscopic treatment of the disease. The peritoneal plasma barrier limits the systemic exposure to the chemotherapy, therefore lowering the systemic toxicity and allowing the intraperitoneal administration of higher doses of chemotherapy compared to the maximum tolerated dose for intravenous administration. The chemotherapy drug (30 mg/m^2 of mitomycin c (Mc) for PC of colorectal origin) is warmed to 42 °C using an extracorporeal circulation system and applied for 60 or 90 min. A pump is used to homogenously distribute the chemotherapy. This treatment results in prolonged survival times, with a median survival of 63 months for patients with resectable and limited PC.[1] The morbidity and mortality rates are 20% and 4%, respectively.

The aim of chemotherapy is to kill cancer cells. The way in which they die will determine the ability of the immune system to recognize the dead cells and to react against them. The concept that apoptosis is tolerogenic, whereas necrosis is immunogenic has been challenged in recent years, as some cases of immunogenic cell death can be caspase-dependent.[3] It is now widely accepted that immunogenic cell death relies on the ability of a specific stimulus to induce the release/exposure of immunogenic signals, classified as damage-associated molecular patterns (DAMPs). The main DAMPs are calreticulin, ATP, the non-histone chromatin-binding protein high mobility group box 1 (HMGB1) and various chaperones from the heat shock protein (HSP) family, notably HSP70 and HSP90.[3,4] The coordinated release of specific DAMPs promotes the recruitment of antigen-presenting cells, which take up material derived from the dead cells and prime the adaptive immune response.[3]

HSPs are a family of highly conserved chaperone proteins that have an important role in proteins folding. It has been described that the intracellular overexpression of HSPs can inhibit cell death.[5] However, HSPs, particularly HSP70 and HSP90, also exhibit potent immunostimulatory activity when exposed on the plasma membrane of stressed or dying cells.[6,7] Indeed, these molecules can be exposed to the outer cellular membrane and can also be released from damaged and viable cells.[8,9] Ecto-HSP70 and ecto-HSP90 act as DAMPs by interacting with a number of

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Received 14 October 2014; revised 20 February 2015; accepted 22 February 2015

antigen-presenting cell-receptors, such as CD91, LOX1 and CD40, and by carrying over tumor peptides that will be recognized by the immune system as tumor antigens.[6]

Taking into account that HIPEC can lead to a long-term protection in patients suffering from PC, the current study was designed to analyze whether the anticancer properties of HIPEC result from the induction of an efficient anticancer immune response. Our results demonstrate that HIPEC through the action of Mc induces an efficient anticancer immune response mediated by the exposure of HSP90 on the plasma membrane of dying cells.

RESULTS

To determine whether HIPEC-mediated cytotoxicity of tumor cells affects the way the immune system reacts to dead tumor cells, we established an *in vitro* model of HIPEC. The current HIPEC procedure for PC of colorectal origin is to combine chemotherapy (mitomycin c, Mc) with hyperthermia (heat shock; HS). This *in vitro* model allowed us not only to analyze if HIPEC is able to induce an anticancer immune response but also to determine what are the respective contributions of Mc and HS on this parameter. Therefore, CT26 cells (a murine colon carcinoma cell line syngeneic to Balb/c mice) were incubated for 90 min at 42 °C (HS), with 100 μM of Mc or with 100 μM of Mc at 42 °C (HIPEC condition, HS+Mc). Then, the culture media was replaced, and the cells were kept in culture for 48 h. Next, the treated CT26 cells were incubated for 24 h with dendritic cells (DCs) derived from Balb/c bone marrow monocytes. As presented in Figure 1a, both the Mc and HIPEC treatments led to DCs activation (as judged by the increase in CD80, CD86 and MHCII expression in DCs), whereas the HS treatment did not. We did not observe an increase in CD40, MHCI expression or in IL12 release (Supplementary Figure 1).

It is well-known that T cells produce IFNγ once antigen-specific immunity develops. To determine whether our activated DCs could activate T cells in a tumor antigen-dependent manner, DCs previously cocultured with treated CT26 cells were incubated with syngeneic naive T cells. The activation ability of these T cells was investigated by measuring interferon-γ production after 10 days of

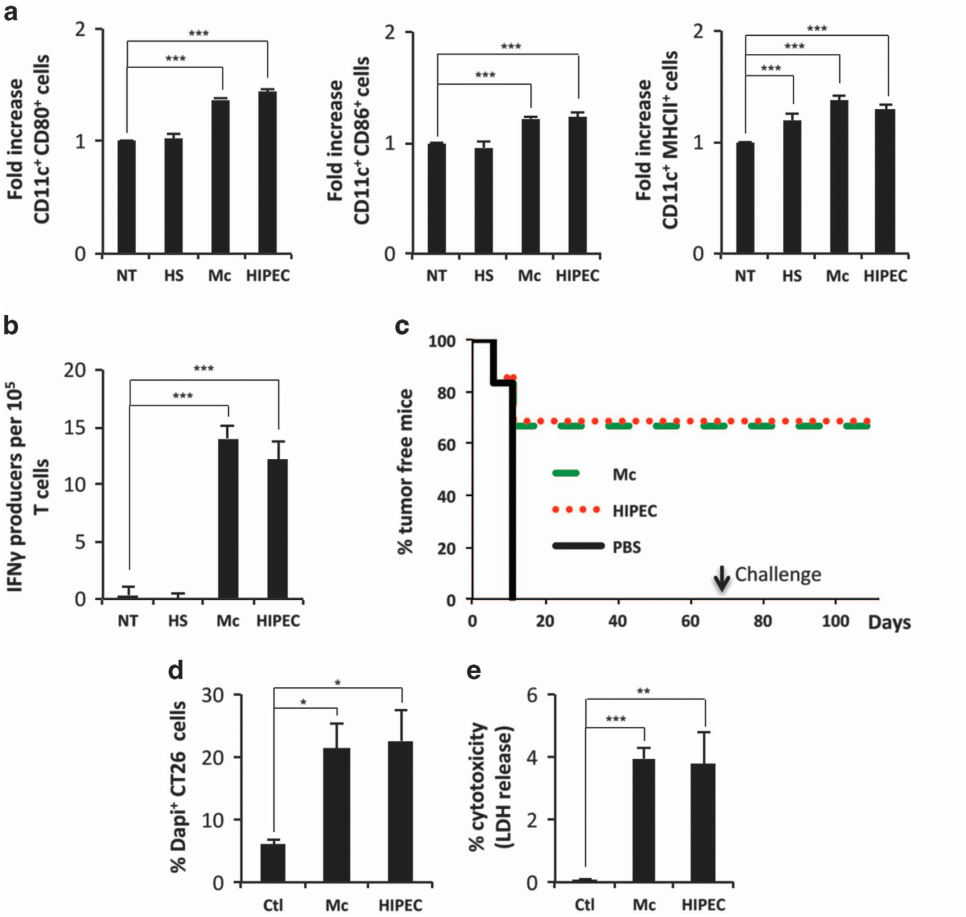

Figure 1. Mc and HIPEC induce a tumor-specific T-cell response. CT26 cells were either non treated (NT) or treated for 90 min with 100 μM of Mitomycin c (Mc); incubation at 42 °C (Heat shock, HS); incubation at 42 °C in the presence of 100 μM Mitomycin c (HIPEC). Media was replaced after treatment. (**a**) 48 h after treatment, CT26 cells were incubated with Balb/c monocyte-derived DCs. Twenty-four hours later, cells were stained for the DC markers CD11c, CD80, CD86 and MHCII, and analyzed by flow cytometry. (**b**) Forty-eight hours after treatment, CT26 cells were incubated with Balb/c monocyte-derived DCs (DC:CT26 ratio 1:2) and then cocultured with naive syngeneic Balb/c T cells (DC:T ratio 1:10). Ten days later, interferon gamma-producing T cells were quantified using ELISPOT. (**c**) Twenty-four hours after treatment, either CT26 cells or PBS were injected subcutaneously into syngeneic Balb/c mice flanks. One week later, live cells were injected into the opposite flank, and the tumor appearance was monitored over time ($n = 6$ mice/group). At day 67, the mice were challenged a second time with live CT26 cells (challenge) and inspected for tumor development. (**d**) CT26 cells or PBS were injected subcutaneously into Balb/c mice. One week later $CD3^+$ cells from PBS or CT26 injected mice were isolated from the spleen and incubated with live CT26 cells. Cell death of CT26 cells was determined by DAPI staining. (**e**) The ability of T cells to kill tumor cells was determined using LDH measurement. Results are expressed as the means ± s.d. and are representative of three different experiments. Each experiment was performed in triplicate. * < 0.05, ** < 0.01 and *** < 0.001.

coculture. Strikingly, both the Mc and HIPEC treatments were highly efficient at activating tumor-specific T cells compared with the HS or control treatments (Figure 1b).

We then reasoned that if Mc and HIPEC treatments are indeed inducing an efficient immune response against tumor antigens, we should be able to vaccinate mice and hence to induce a protective anticancer immune response. Syngeneic immunocompetent Balb/c mice were subcutaneously injected with PBS or with either Mc- or HIPEC-treated CT26 cells into one flank. One week later, the mice were challenged by injecting viable CT26 cells into the other flank. It should be noted that the HS treatment of CT26 could not be tested using vaccination assays as it does not kill the cells. Interestingly, the mice injected with either Mc- or HIPEC-treated CT26 cells exhibited a vigorous antitumor immune response *in vivo* (Figure 1c) under conditions that led the control mice to develop tumors in 10 days after the challenge. On rechallenge with live tumor cells at day 67, the vaccinated mice did not develop tumors for another 40 days, indicating the establishment of a permanent antitumor immune response. Finally, we confirmed that only T cells isolated from vaccinated mice (that is, Mc- and HIPEC-treated cells) were able to kill CT26 tumor cells *ex vivo* (Figures 1d and e).

Thus, we conclude that HIPEC treatment of cancer cells lead to an efficient antitumor immune response. In addition, we made the unexpected observation that Mc was as efficient as HIPEC to induce such effect.

Mc- and HIPEC-treated cells are killed in a similar manner

We further analyzed the way the tumor cells died in response to the Mc and HIPEC treatments. The cells were treated as shown in Figure 2a and cell death was analyzed 24, 36 and 48 h later. We observed that the Mc and HIPEC treatments, but not the HS treatment, led to significant caspase activation (Figure 2b). Both the Mc and HIPEC treatments induced a similar level of cell death, which was partially prevented by the use of a pan-caspase inhibitor, q-VD-OPH (Figure 2c). These results were confirmed by DNA-fragmentation analysis during cell death (Figure 2d). In addition, we did not observe any induction of necroptosis in our model (Supplementary Figure 2).

It has recently been suggested that hyperploid cancer cells can become immunogenic;[10] therefore, we investigated whether our treatments were modifying the proportion of hyperploid tumor cells. As shown in Figure 2e, regardless of the time of analysis, none of the treatments significantly modified the proportion of hyperploid cells.

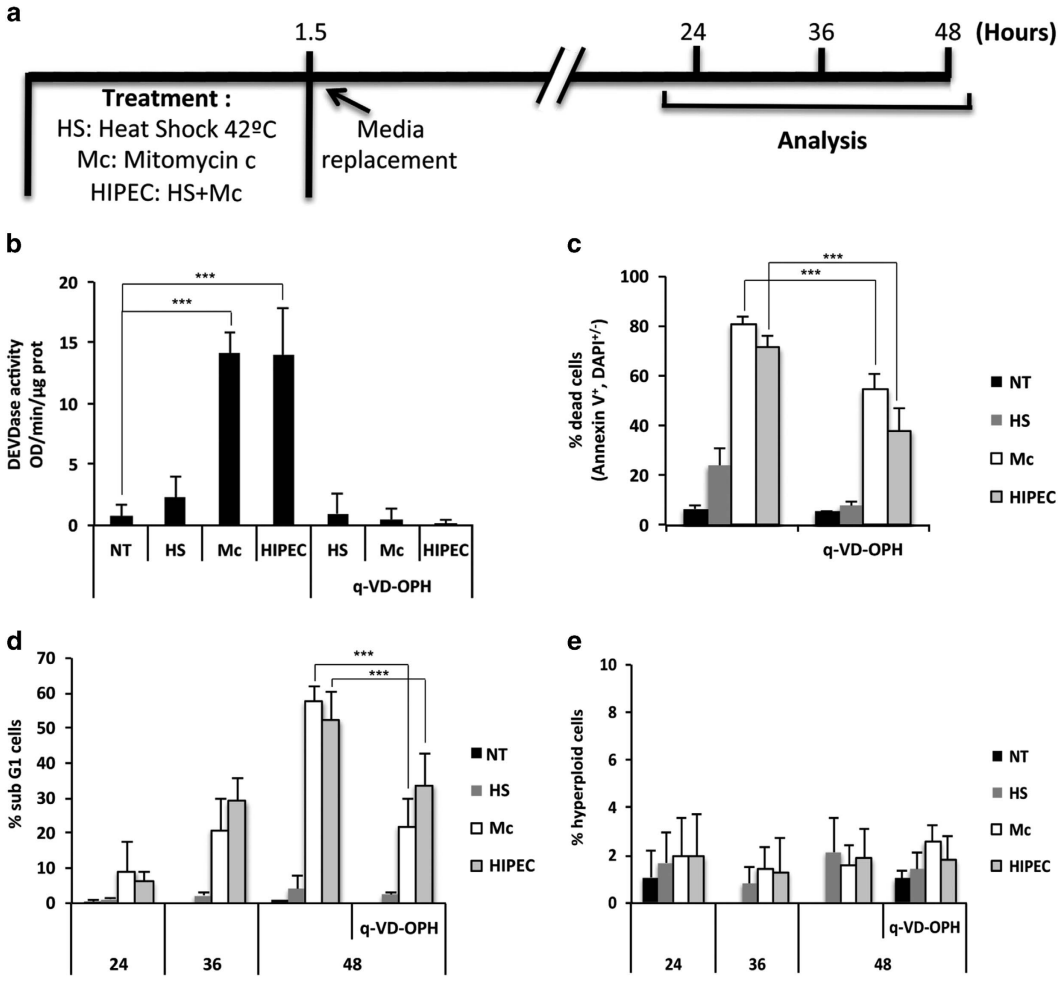

Figure 2. Mc and HIPEC treatments lead to a similar extent of cell death and do not increase the proportion of polyploid cells. (**a**) Schematic representation of the different treatments used *in vitro*. (**b**) CT26 cells were treated as described in Figure 1. In some conditions, a pan-caspase inhibitor (20 µM of q-VD-OPH) was added during the experiment. DEVDase activity was measured 48 h after the treatment. (**c**, **d**, **e**) CT26 cells were treated as in Figure 1, either with or without q-VD-OPH. Cell death was determined by flow cytometry using Annexin V/DAPI staining (**c**), by analyzing DNA-fragmentation (**d**) or by analyzing polyploid cells (**e**). Results are expressed as the means ± s.d. from two independent experiments performed in triplicate. ***$P < 0.001$.

We therefore concluded that the Mc and HIPEC treatments induce antitumor vaccination to the same extent without modifying the kinetics of cell death or increasing the proportion of hyperploid cells.

HIPEC-induced antitumor vaccination is mediated by HSP90

Knowing that (i) hyperthermia during the HIPEC procedure induces HSPs expression[11] and (ii) the surface exposure of HSPs on tumor cells participate in the anticancer immune response,[4] we investigated the expression of HSPs in response to HS, Mc or HIPEC treatment. mRNA level analysis by quantitative PCR revealed that HS and HIPEC induced the expression of all the HSPs, as expected (Figure 3a). HSP90B1 corresponds to endoplasmin protein (also known as 94 kDa glucose-regulated protein), HSP90AB1 corresponds to HSP90-beta protein (also named HSP90-beta, HSP 84, TSTA) and HSP90AA1 corresponds to HSP90-alpha protein (also named HSP90-alpha, HSP 86, 86 kDa). We also determined that Mc could lead to an increase in the expression of all isoforms of HSP90 mRNAs, albeit at latter times (Supplementary Figure 3).

We then assessed global protein expression of HSPs response to HS, Mc or HIPEC treatments. We observed that HSP90 expression was increased on treatments compared with the control condition (Figure 3b).

When we monitored the exposure on the surface of HSPs on live CT26 cells in response to each treatment, we observed that both the Mc and HIPEC treatments resulted in the plasma membrane exposure of some (HSP40, HSP90 and HSP70 in the case of Mc and HIPEC treatment) but not all HSP members, suggesting some specificity in the effect (Figure 3c). We then

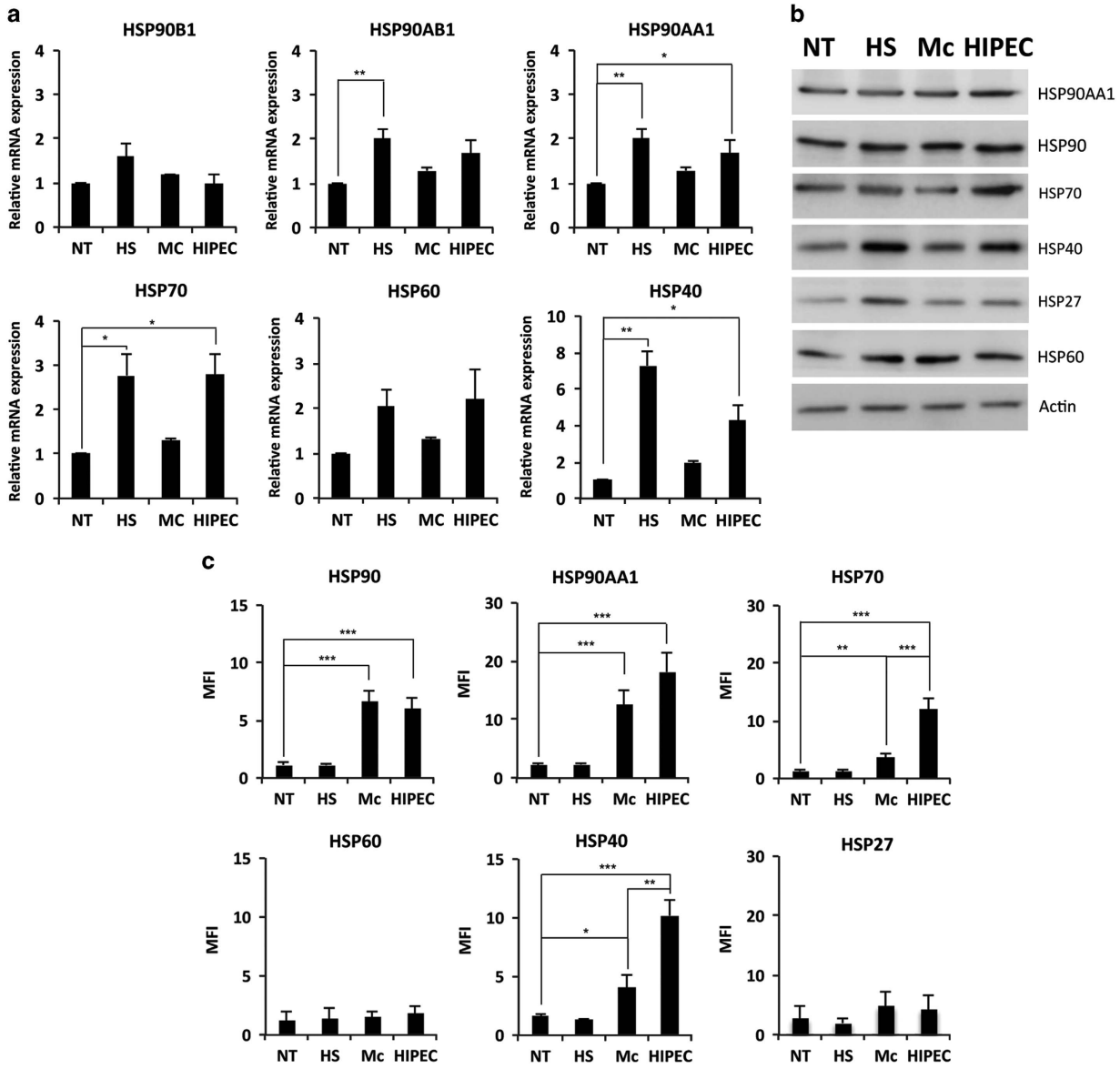

Figure 3. Both Mc and HIPEC induce plasma membrane exposure of HSP90. (**a**) CT26 cells were treated as described in Figure 1. Ninety minutes after treatment the mRNA levels of several HSPs were measured by real-time PCR. (**b**) Twenty-four hours after treatment CT26 cells were lysed and analyzed by western blot. Protein expression levels of HSPs were determined. Actin was used as a loading control. (**c**) Forty-eight hours after treatment CT26 cells were collected, and the exposure of HSPs on the plasma membrane was analyzed by flow cytometry in DAPI-negative cells. Results are expressed as the means ± s.d. from three independent experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; when not specified, the difference is not significant.

verified by immunofluorescence staining that HSP90 could be observed on the plasma membrane of HIPEC- and Mc-treated cells while it is found diffused in the cytosol of untreated or HS-treated cells (Supplementary Figure 4).

To uncover the potential role of HSP70 and HSP90 membrane exposure in the HIPEC-mediated anticancer immune response, we analyzed DCs activation when the cells were fed with HS-, Mc- or HIPEC-treated CT26 cells in the presence of specific HSP70- or HSP90-blocking antibodies[7,12] or a control anti-IgG antibody. In contrast to the anti-HSP70 antibody, the HSP90-blocking antibody was very efficient to prevent DCs activation (Figure 4a, as previously suggested[13]), indicating that HSP90 is involved in the HIPEC-mediated anticancer vaccination effect. Similar results were obtained using another anti-HSP90 antibody (Supplementary Figure 5).

To further support this observation, we used 17-AAG, an HSP90 inhibitor, and VER155008, an HSP70 inhibitor, and analyzed DCs activation. HSP90 inhibition using 17-AAG, but not HSP70 inhibition using VER155008, limited DCs activation after coculture with previously treated CT26 cells (Figure 4b). We verified that none of the inhibitors affected the HIPEC-mediated cytotoxicity (Supplementary Figure 6A). We verified that an efficient dose of each inhibitor was being used (Supplementary Figure 6B) by assessing the ability of 17-AAG and VER155008 to decrease c-Raf expression in Mc and HIPEC conditions compared with non-treated condition, as cRAF is an important HSP90 client protein.[14,15]

We then addressed the role of Mc- and HIPEC-mediated HSP90 exposure in inducing the anticancer immune response *in vivo* by using mice vaccination assays. CT26 cells were treated with Mc (Figure 4c) or with HIPEC (Figure 4d) in the presence of either 17-AAG, VER155008 and then injected into immunocompetent syngeneic Balb/c mice. We also included a condition where the treated cells were incubated in the presence of an anti-HSP90 antibody and then injected into Balb/c mice. One week later, the mice were then challenged as shown in Figure 1c. Mc and HIPEC-treated cells were equivalent in inducing the vaccination of the mice. Interestingly, 17-AAG or anti-HSP90 antibody but not VER155008 treatment diminished the induction of the anticancer

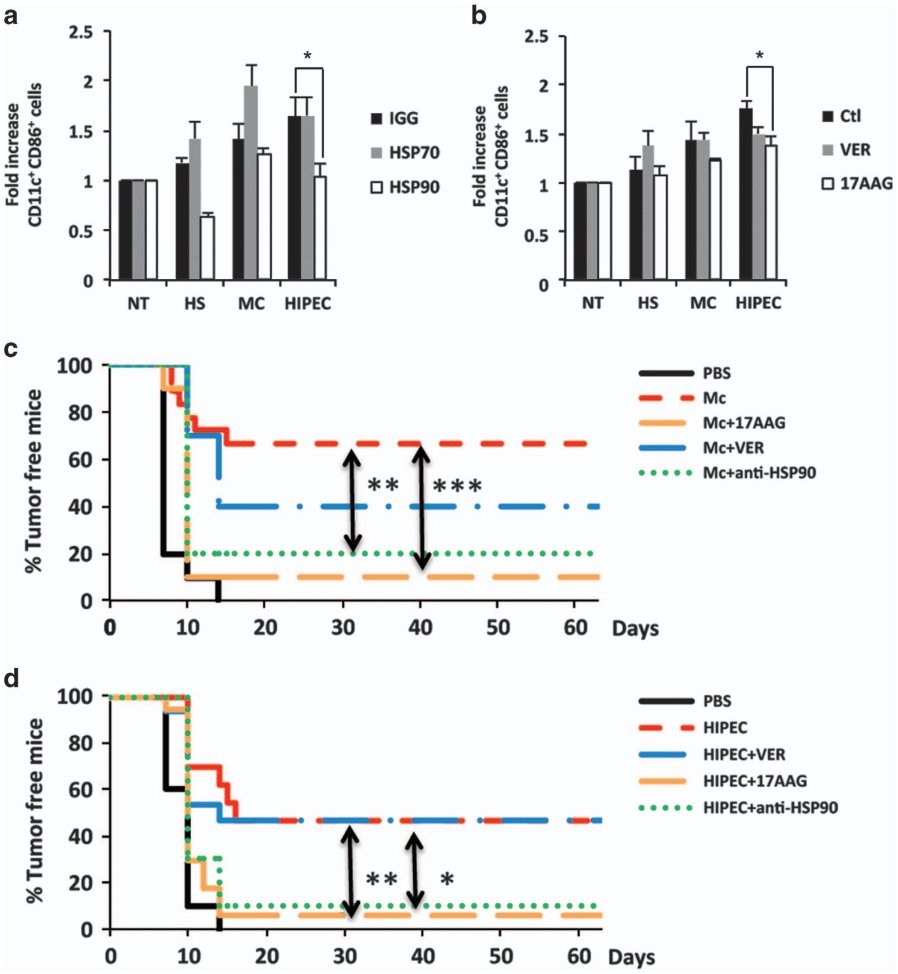

Figure 4. HSP90, but not HSP70, is implicated in HIPEC-mediated tumor-specific immune response. (**a**) CT26 cells were incubated with either control anti-IgG (IGG), anti-HSP70 or anti-HSP90 antibodies (1 μg/ml) for 1 h before and during the coculture. (**b**) HSP inhibitors, 17 AAG (0.1 μM) or VER155008 (VER, 5 μM) were added 1 h before, during, after the treatment and during the coculture. Treated cells were then incubated with Balb/c monocyte-derived DCs. Forty-eight hours later, cells were stained for CD11c and CD86 and analyzed by flow cytometry. CT26 cells were incubated with either PBS, VER, 17-AAG or anti-HSP90 as in **a** and then treated with Mc (**c**) or HIPEC (**d**). Either treated cells or PBS were then injected subcutaneously into syngeneic Balb/c mouse flanks. One week later, live cells were injected into the opposite flank, and the tumor appearance was monitored over time ($n = 5$ mice for the PBS group; $n = 13$ for the HIPEC group; $n = 17$ for the HIPEC+17-AAG group; $n = 15$ for the HIPEC+VER group; $n = 18$ for the Mc group; $n = 10$ for all the other groups). Results are expressed as the means ± s.d. and are representative of two or three independent experiments. Each experiment was performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, when not specified, the difference is not significant.

immune response, further suggesting that HSP90 but not HSP70 is involved in the observed effect.

Together, our data suggest that Mc and HIPEC can induce an anticancer immune response at least in part through the plasma membrane exposure of HSP90.

DISCUSSION

The most effective therapeutic approaches specifically kill tumor cells in a way that induces an efficient immune response against tumor antigens, and patients who exhibit a strong immune response against their tumor show prolonged survival.[16] Therefore, there is considerable interest in understanding the biochemical features of immunogenic versus non-immunogenic tumor cell death induced by anticancer therapies. A major challenge is to safely restore the antitumor immune response by using chemotherapeutic treatments that can induce an immunogenic type of cell death[17] and that can limit the resurgence of the disease. Here, we showed that the HIPEC procedure is not only able to kill tumor cells but also able to induce an efficient anticancer immune response. We demonstrated that this immune response is mediated at least in part by the exposure of HSP90 to the plasma membrane. To our knowledge, this is the first example of a surgical/chemotherapeutic treatment that is able to induce an antitumor immune response that may participate in the long-term protection of treated patients.

To discriminate between the effects of HS, Mc or HIPEC treatment on tumor cells, we established an *in vitro* model for HIPEC. Interestingly, our work revealed that both Mc and HIPEC similarly induce immunogenic cell death, which results in anticancer vaccination (Figures 1, 2 and 4), indicating that Mc treatment in this setting does not require hyperthermia to induce efficient anticancer vaccination (Figures 1 and 4). This is an interesting observation, as it is known that the addition of HIPEC to cytoreductive surgery increases the risk of morbidity and mortality.[18] On the other hand, previous studies have demonstrated that hyperthermia increases the cytotoxicity of cytostatic drugs such as oxaliplatin, Mc, doxorubicin, irinotecan or cisplatin by enhancing the accessibility and penetration of the chemotherapy to the tumor tissues/isolated tumor cells, which may be relatively resistant to systemic chemotherapy, therefore increasing their antitumor effects. This has been suggested to be involved in the beneficial effect observed on patients that undergo HIPEC treatment.[19] Thus, the favorable effects of this procedure may lie in both an enhancement of penetration of the drug by hyperthermia and the effective induction of an antitumor immune response by Mc. It seems therefore important to validate in patients the beneficial effects of hyperthermia and of local chemotoxicity through the launch of a large-scale clinical trial between several national or international centers to definitively validate which procedure is the best for the patients. However, to the best of our knowledge, such studies have not been conducted.

Immunogenic cell death is characterized by the early surface exposure of HSPs on the cell surface or in proximity to dying cancer cells, resulting in the formation of tumor antigen-HSP complexes, which are processed by DCs to cross-prime T cells more efficiently than tumor antigens alone.[20] In the present study we linked the immunogenicity of HIPEC- and Mc-treated cells to HSP90 exposure on the cell surface. However, the mechanism leading to HSP90 exposure remains unknown. HIPEC- and Mc-linked lesions in the membranes of treated cells may cause the inner membrane sections to flip to the outer surface[21] and expose HSP molecules associated with membrane proteins or lipids. In addition, surface-localized HSPs may have a role in the stabilization of damaged membranes and preserve their integrity.[22]

Several HSP90 inhibitors are currently in clinical trials in oncology.[23] Others have shown that the inhibition of HSP90 can have an impact on DCs maturation. Indeed, as HSP90 has a role in the assembly and folding of proteins, its inhibition can affect the tertiary structure of proteins as well as proteins degradation, causing a decrease in DCs maturation.[13] Our results in *in vivo* vaccination assays indicate that HSP90 inhibitors may unexpectedly affect not only DCs cells but also the membrane expression of 'find-me' signals like HSP90 by dying cancer cells. This would alter the antigen uptake and processing by DCs having a negative effect on the anticancer immune response. These notions should be now carefully considered for further development of HSP90 inhibitors.

The observation that HIPEC can lead to the immunogenic death of tumor cells not only helps reveal how HIPEC protects patients in a long-term basis but also provides the rational for using immunomodulating approaches in the treatment of PC, therefore opening new therapeutic options. A key element for developing new therapies is to safely activate the anticancer immune response. The development or identification of novel compounds that could safely induce HSP90 exposure on the plasma membrane of cancer cells would be of great interest. However, no such compounds have yet been discovered. Another interesting option would be to activate immunogenic cell death through the exposure/release of other DAMPs. In this sense, we recently established that a glycolytic inhibitor can induce an anticancer immune response via the cell surface exposure of calreticulin.[24] Therefore, the possibility of including such metabolic inhibitors during the HIPEC procedure is worth investigating and this might yield an innovative development in the future. As the peritoneal plasma barrier limits the systemic concentration of the chemotherapy, a large amount of metabolic inhibitors could potentially be used during HIPEC to maximize the chemotherapy-induced toxicity and to further enhance the induction of immunogenic cell death, with minimal systemic toxicity. In addition, although designed for neoplastic pathologies, this strategy may be extended to intra-thoracic hyperthermic chemotherapy, which is used for the treatment of various pleural malignancies.[25]

MATERIALS AND METHODS

Cell culture and cell death assays

CT26 cells were obtained from ATCC and cultured as recommended. To induce cell death, CT26 cells were treated for 1.5 h with one of the following conditions: (1) 100 μM Mc (Tocris Bioscience, Bristol, UK); (2) HS at 42 °C; or (3) HS at 42 °C in the presence of 100 μM Mc (HIPEC). The cells were harvested 24, 36 or 48 h after treatment, stained with Annexin V (BD Biosciences, Le Pont de Claix, France) and 4',6-diamidino-2-phenylindole (0.5 μg/ml, Molecular Probes, Grand Island, NY, USA) and analyzed immediately by flow cytometry using a MACS-Quant Analyzer (Miltenyi Biotec, Paris, France). In some cases, the tumor cells were incubated for 1 h before, during and after the treatment with 20 μM q-VD-OPH (SM Biochemicals, Anaheim, CA, USA), 0.1 μM 17-AAG (Tocris Bioscience) or 5 μM VER155008 (Tocris Bioscience) prior to analysis. For mice vaccination experiments CT26 cells were incubated with anti-HSP90 (Enzo Life Sciences, Farmingdale, NY, USA) antibody 1 h before treatment.

For the lactate dehydrogenase (LDH) release experiments, CT26 cells were treated as indicated, and after 3 h, 2×10^6 cells were subcutaneously injected into syngeneic, immunocompetent 4-week-old Balb/c mice. One week later, $CD3^+$ cells were isolated from the mouse spleens using autoMACS (Miltenyi Biotec). The resulting purified cells were co-incubated with CT26 cells at a ratio 1:5 in the presence of IL-2 (0.1 ng/ml, AbD Serotech, Kidlington, UK) for 4 h at 37 °C. LDH release was then measured using a colorimetric assay kit according to the manufacturer's instructions (Roche, Indianapolis, IN, USA).

In parallel, flow Cytometry (MACS-Quant Analyzer Miltenyi Biotec) was used to analyze the cell viability of CT26 cells. The gating was made on the CD3-negative population and back gating was used to confirm the difference in forward scatter and side scatter parameters between lymphocytes and CT26 cells. Cell death was evaluated by looking at plasma membrane permeabilization of CT26 cells using 4',6-diamidino-2-phenylindole staining.

Detection of HSPs on the cell surface

Cells were collected and stained with rabbit anti-mouse HSP antibodies (1:200, see 'western blot' section) for 30 min at 4 °C in 2% fetal calf serum–phosphate-buffered saline, then incubated for 30 min with an anti-rabbit Alexa Fluor 488-conjugated secondary antibody (1:200, Molecular Probes). The pellet was re-suspended in phosphate-buffered saline buffer with 4',6-diamidino-2-phenylindole (0.5 μg/ml, Molecular Probes). The samples were then analyzed on a MACS-Quant Analyzer (Miltenyi Biotec) for HSP staining in DAPI-negative cells.

Antitumor vaccination

All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee and of the regional ethics committee (approval reference NCE/2011-35).

CT26 cells were treated as indicated, and after 24 h, 2×10^6 cells were subcutaneously injected into syngeneic, immunocompetent 4-week-old Balb/c mice. One week later, the mice were challenged with injection of 0.5×10^6 live cells into the contralateral flank. The animals were then inspected three times a week for tumor development. Increases in tumor size were measured using a caliper. At day 67, the mice were challenged a second time with injection of 0.5×10^6 live cells into the contralateral flank. See reference 26 for more details.

DCs preparation, activation and interferon gamma measurement

Bone marrow cells were flushed from the femurs and tibias of young Balb/c wild-type mice, and monocytes were isolated using an autoMACS (Miltenyi Biotec) and incubated with murine granulocyte-macrophage colony-stimulating factor (100 ng/ml, Miltenyi Biotec) and IL-4 (100 ng/ml, AbD Serotech) for 1 week in Iscove's modified dulbecco's medium containing 10% inactivated fetal calf serum, antibiotics and 50 μM β-mercaptoethanol. CT26 cells were treated as described and cultured for 48 h after treatment. Immature DCs were then fed with treated CT26 cells at a DC:tumor cell ratio of 1:2.

For the analysis of DCs activation, the cells were cocultured for 24 or 48 h. Then, the cells were stained with anti-MHCI, anti-CD40 and anti-CD11c (1:200, BD Biosciences) for 30 min at 4 °C in 2% fetal calf serum–phosphate-buffered saline. The samples were then analyzed using a MACS-Quant Analyzer (Miltenyi Biotec). Control IgG (R&D systems, Lille, France) or anti-HSP90 (Cell Signaling, Denvers, MA, USA) antibodies were added 1 h before the coculture.

Either 17-AAG (0.1 μM, Tocris Bioscience) or VER105008 (5 μM, Tocris Bioscience) were added before, during and after the CT26 treatment and during the coculture.

T cells from the spleen of naive syngeneic mice were sorted using an autoMACS Pro Separator (Miltenyi Biotec) and added to tumor-loaded DCs at a ratio of 1:10 in the presence of IL-2 (0.1 ng/ml, Abd Serotech).

Co-cultures were tested for the presence of interferon gamma-producing T cells 10 days after the stimulation with DCs using an enzyme-linked immunospot assay (BD Bioscience) on T cells.

Quantitative reverse transcription-PCR analysis

Total RNA was isolated from cells using the RNeasy Micro Kit (Qiagen, Paris, France) according to the manufacturer's protocol. After reverse transcription-PCR, the relative mRNA expression level of mouse HSP90AA1, HSP90AB1, HSP90B1, HSP70, HSP60 and HSP40 were obtained by real-time quantification PCR, using the TaqMan PCR Master Mix (Eurogentec, Seraing, Belgium) and TaqMan assay primer set (Applied Biosystems, Foster City, CA, USA) on the 7500 Fast and the Step One (Applied Biosystems) according to the manufacturer's instructions. The house-keeping gene 18 s was used as a control for RNA quality, and used for normalization.

DEVDase activity

To assess DEVDase activity, the cells were lysed in buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10 mM pH 7.4, NaCl 150 mM, EDTA 5 mM, 1% NP40, 10 μg/ml aprotinin, 1 mM PMSF, 10 μM leupeptin) 48 hours after treatment. Lysates were standardized for protein content and loaded into a black 96-well plate (CellStar, St Louis, MO, USA) in the presence of 0.2 mmol/l of the caspase-3 substrate Ac-DEVD-AMC diluted in the following buffer: 50 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 150 mmol/l NaCl, 20 mmol/l EDTA and 10 mmol/l dithiothreitol. Caspase activity was determined both with and without the presence of 1 μmol/l of the caspase-3 inhibitor Ac-DEVD-CHO using a fluoroscan at 460 nm, and the specific activity was expressed as the change in absorbance per minute per milligram of protein.

Statistical methods

Data are expressed as the means ± s.d. Differences in the calculated means between the groups were assessed by two-sided Student's *t*-tests. Tumor-free survival time was calculated as the time from subcutaneous tumor cell injection until one of the following events: (1) the first occurrence of a tumor with a volume of 1500 mm^3, (2) death from any cause or (3) until 60 (or 112) days of follow-up without any clinical events. Kaplan–Meier analyses were performed, and the curves were compared using log-rank tests. A *P*-value < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We gratefully acknowledge the Centre Méditerranéen de Médecine Moléculaire animal room and imaging facilities. We thank Jozef Bossowski, Drs Raucoules and Benchimol, Benjamin Lefebvre and all of the operating room and surgical digestive service nurses for their help. This work was supported by the Fondation ARC (Association pour la Recherche sur le Cancer), the Agence Nationale de la Recherche (LABEX SIGNALIFE ANR-11-LABX-0028-01). CRP is supported by the Fondation ARC, LM is supported by la Ville de Nice and by Fondation pour la Recherche Medicale (FRM) and JC is supported by la Fondation de France.

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