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Hyperthermic treatment at 56 °C induces tumour-specific immune protection in a mouse model of prostate cancer in both prophylactic and therapeutic immunization regimens

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ABSTRACT

Most active cancer immunotherapies able to induce a long-lasting protection against tumours are based on the activation of tumour-specific cytotoxic T lymphocytes (CTLs). Cell death by hyperthermia induces apoptosis followed by secondary necrosis, with the production of factors named "danger associated molecular pattern" (DAMP) molecules (DAMPs), that activate dendritic cells (DCs) to perform antigen uptake, processing and presentation, followed by CTLs cross priming. In many published studies, hyperthermia treatment of tumour cells is performed at 42–45 °C; these temperatures mainly promote cell surface expression of DAMPs. Treatment at 56 °C of tumour cells was shown to induce DAMPs secretion rather than their cell surface expression, improving DC activation and CTL cross priming *in vitro*.

Thus we tested the relevance of this finding *in vivo* on the generation of a tumour-specific memory immune response, in the TRAMP-C2 mouse prostate carcinoma transplantable model. TRAMP-C2 tumour cells treated at 56 °C were able not only to activate DCs *in vitro* but also to trigger a tumour-specific CTL-dependent immune response *in vivo*. Prophylactic vaccination with 56 °C-treated TRAMP-C2 tumour cells alone provided protection against TRAMP-C2 tumour growth *in vivo*, whilst in the therapeutic regimen, control of tumour growth was achieved combining immunization with adjuvant chemotherapy.

1. Introduction

In developed countries, prostate cancer (PCa) is the most common male malignancy [1–3]. If diagnosed prior to metastases formation it may have a long natural course, with a window of therapeutic intervention. So far, the cytotoxic chemotherapy drugs showing survival advantage for PCa are docetaxel and carbazitaxel [1]. Anticancer chemotherapy, as radiotherapy, is largely mediated by apoptosis of neoplastic cells. Therapy-induced apoptotic tumour cell death usually progresses to secondary necrosis, increasing tumour cells immunogenicity when injected into immune-competent mice [4–6]. Such enhanced immunogenicity relies on the expression of danger associated pattern (DAMP) molecules (DAMPs), like high mobility group box 1 (HMGB1) [7]. HMGB1 activates inflammation and can act as cytokine itself [8]. Chemotherapeutics such as docetaxel exert cytokine-like immune-stimulatory properties, improving tumour-protective immune responses as well [9].

Hyperthermia treatments such as high-intensity focused ultrasound (HIFU), radiofrequency ablation (RFA), irreversible electroporation (IRE) and microwave ablation (MWA) have emerged as new ablative treatments for solid tumours. These techniques raise local tissue temperatures up to 45–85 °C, generating lesions with a central zone at temperatures higher than 60 °C where protein denaturation occurs, causing coagulative necrosis, surrounded by a transitional zone of sub-lethal hyperthermia (40–60 °C) where cells undergo apoptosis or recover from injury [10]. Inflammatory

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infiltrates were found both in the transitional zone and in distant, untreated tumour metastases, suggesting that ablation induced an overall immune activation, although often too modest to completely eradicate established tumours [10]. These temperatures cause tumour cell death with generation of apoptotic material that can proceed to secondary necrosis and contains DAMPs activating DC functions of antigen uptake, processing and cross-priming of cytotoxic T cells (CTLs) [10,11]. The therapeutic efficacy of hyperthermia joined to radiotherapy was investigated also in PCa patients [12–14].

In a mouse PCa transplantable tumour model, intra-tumour injection of immature DCs (iDCs) after local hyperthermia treatment of tumour at 42–45 °C induced tumour-specific immunity with statistically significant tumour growth reduction [15]. Tumour cells exposure at 42–45 °C increased intracellular DAMP heat shock protein (HSP) 70; temperature raise to 56 °C determined HSP70 and HMGB1 secretion, that strongly improved DCs activation by reducing iDCs-maturation time and reverting the immunosuppressive action of tumour cells on CTL cross priming [16]. Thus, tumour treatment at 56 °C was more active than that at 42–45 °C in inducing tumour cells endocytosis and T cell activation by iDCs and T cell activation [17].

In the present report, the treatment at 56 °C was applied to study both its ability to increase *in vitro* the immunogenicity of several human and mouse tumour cell lines of different origin and to improve *in vivo* the vaccination efficacy exploiting the transgenic adenocarcinoma of the mouse prostate (TRAMP)-C2 transplantable tumour model [18] either in prophylactic or therapeutic regimen. Docetaxel-based adjuvant chemotherapy was combined with immunization. In this tumour model we have previously demonstrated that interferon-γ (IFN-γ) is critical for inducing a tumour protective T-cell-mediated immune response, by simultaneously increasing MHC-I-mediated antigen presentation and antagonising tumour-secreted TGF-β effects [19].

2. Materials and methods

2.1. Cell lines

TRAMP-C1 (ATCC-CRL-2730) and TRAMP-C2 (ATCC-CRL-2731), a gift of N. Greenberg (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) [18] and T1525 cell line [20] were cultured and treated with mouse IFN-γ as described [19]. TS/A [21], N202-1.A [22] LM5 (NT-2) [23] are mouse mammary adenocarcinoma cell lines; CT26 (ATCC-CRL--2638), C26 and C51 [24] are mouse colon carcinoma cell lines, all cultured in DMEM with 10% FBS. Human cell lines, all cultured in RPMI supplemented with 10% FBS are: MCF-7 (ATCC-HTB-22), MDA-MB-468 (ATCC-HTB-132) and MDA-MB-231 (ATCC-HTB-26) from mammary adenocarcinoma; HCT-15 (ATCC-CCL-225), LoVo (ATCC-CCL-229) and HT-29 (ATCC-HTB-38) from colon carcinoma; LNCAP clone FGC (ATCC-CRL1740), DU145 (ATCC-HTB-81) and PC3 (ATCC-CRL-1435) from prostatic carcinoma. All cell cultures were incubated at 37 °C in 5% CO_2 atmosphere.

2.2. Generation of DCs

Immature DCs (iDCs) were obtained from the bone marrow (BM) of C57BL/6J mice according to standard protocols [25] by a 6-day incubation of BM-derived cells in 6-well tissue culture plates (GREINER Labortechnik, Germany) in complete medium supplemented with murine GM-CSF (final concentration: 20 ng/ml) and murine IL-4 (final concentration: 100 ng/ml) (PEPROTECH, Rocky Hill, NJ, USA). On day 7th, non-adherent and loosely adherent cells were collected and used as iDCs.

2.3. Hyperthermic treatment and endocytosis assay

Human and murine tumour cell lines in the logarithmic growth phase, resuspended in fresh culture medium at the concentration of 25×10^6/ml, were heated at 56 °C for 30 min and then incubated at 37 °C for 4 h [16]. One aliquot of the cell suspension was assayed for cell death by Annexin V-FITC/PI-PE kit (ROCHE Diagnostics Corporation, Indianapolis, IN, USA), and analysed by flow cytometry. More precisely, the percentages of early apoptotic ($AnnV^+/PI^-$), primary necrotic ($AnnV^-/PI^+$) and late apoptotic (secondary necrotic) ($AnnV^+/PI^+$) cells were assessed.

For endocytosis assay, tumour cells red-stained with PKH26-PE (SIGMA, St. Louis, MO, USA) according to the manufacturer's instructions were treated 30 min at 56 °C, 4 h at 37 °C, then mixed with iDCs (2:1 ratio) in murine GM-CSF- and IL-4-containing medium. Following a 24 h incubation at 37 °C, cell mix was stained with anti-CD11c (clone N418, BD Biosciences, Bedford, MA, USA). DCs endocytosis was assessed by flow cytometry as the percentage of double-stained cells [16]. iDCs activation was evaluated as increase of MHC-II and CD80/86 expression by flow cytometry with anti-mouse MHC II (M5/114.15.2), CD80 (16-10A1) and CD86 (GL1) antibodies after co-culture with 56 °C-treated or untreated TRAMP-C2 cells or stimulation with the bacterial endotoxin lipopolysaccharide (LPS) (2 μg/ml) used as positive control of iDC activation [26,27]. In order to confirm iDCs activation, the supernatant of the same co-cultures described above was collected and interleukin (IL)-12p70, tumour necrosis factor (TNF)-α, interferon (IFN)-α and chemokine (C-X-C motif) ligand (CXCL)-10 cytokines were quantified by a custom multicytokine ELISA (ProcartaPlex, ThermoFisher, Waltham, Massachusetts, USA).

2.4. Confocal laser scanning microscopy

iDCs were immunomagnetically enriched by CD11c microbeads (Miltenyi Biotec Bergisch Gladbach, Germany) and labelled with carboxyfluorescein succinimidyl ester - CFSE (Thermo Fisher, Waltham, Massachusetts, USA) according to manufacturer's instructions. CFSE labelled iDCs were co-cultured with PKH26-PE stained TRAMP-C2 as already described for endocytosis assay. After 24 h cells were washed, placed on glass-slides by cytospin and processed for confocal microscopy. Images were acquired on a Leica TCS SP5 AOBS system (Leica Microsystems) with a 63x oil immersion objective, employing a diode laser at 405 nm for Hochest, an Ar laser at 488 nm for CFSE and a He/Ne laser at 543 for PKH26-PE. Images were analysed with LEICA LAS X software.

2.5. ELISA assays

Supernatants derived from tumour cells, treated or not at 56 °C for 30 min and then incubated at 37 °C for 4 h, were tested by ELISA to determine the release of HMGB1, according to manufacturer's instructions (IBL INTERNATIONAL GMBH, Hamburg, Germany).

Spleen cells of tumour bearing mice were *in vitro*-stimulated by 5 days-co-culture with γ-irradiated (6000 cGy) TRAMP-C2 cells and the levels of IFN-γ produced were evaluated by a mouse IFN-γ ELISA according to manufacturer's instructions (ARCUS BIOLOGICALS, Modena, Italy).

2.6. In vivo experiments

In vivo experiments were performed with two-months-old C57BL/6J male mice (Charles River Italia, Calco, Lecco, Italy). A dose of 5×10^6 TRAMP-C2 cells, injected subcutaneously (s.c.) in a volume of 0.2 ml demonstrated to be the minimal dose resulting in TRAMP-C2 growing tumours in 100% of injected syngeneic animals

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at 30 days from s.c. inoculum [18,19], and thus it was chosen as tumorigenic dose in the present study. The same dose of 56 °C-treated TRAMP-C2 cells was used as vaccine. For this purpose, cells were re-suspended in fresh culture medium at the concentration of 25×10^6/ml before 56 °C-treatment, to obtain 0.2 ml/cell suspension aliquots containing $5x10^6$ cell each, ready for s.c. inoculation, to avoid further manipulations. When indicated, some groups of mice were also injected s.c. either with 5×10^6 56 °C-treated TRAMP-C2 cells washed and re-suspended in fresh medium after 56 °C-treatment, or with DCs (1×10^6/mouse) loaded with 56 °C-treated necrotic TRAMP-C2 cells [28]. In therapeutic protocols, 13 days post s.c. inoculation of alive TRAMP-C2 cells, selected groups received docetaxel preconditioning (20 mg/kg) by intraperitoneal (i.p.) administration. All animals bearing tumours were euthanized when the tumour mass reached a volume of 1500 mm^3, or became ulcerated or as soon as signs of pain and fatigue were perceived. Volumes of sub-cutaneous growing tumours were measured with callipers twice a week until 60 days from challenge, chosen as experimental endpoint because in untreated mice at this time almost all tumours have reached a volume of 1500 mm^3. The following formula was used to calculate tumour mass: $(d_L \times d_W \times d_H)/2$, where d_L, d_W and d_H are the three diameters of the mass (length, width and height) [29]. All *in vivo* experiments were carried out following the EU Directive 2010/63/EU for animal experiments and are part of a Research Protocol approved by the Italian Ministry of Health, Decree n. 16/2012-B.

2.7. Statistics

Survival analysis was carried out using the Kaplan-Meier estimator, a non-parametric statistics developed by Kaplan and Meier [30], and the log-rank test to compare survival of differently treated-groups [31]. Differences in tumour incidence were also assessed using χ^2 test with Yates correction and were considered statistically significant for P values ≤ 0.05. Statistical calculations were performed using Stata™ IC v.10.0 (StataCorp, Texas, USA) for Microsoft Windows®. Statistic analysis for *in vitro* experiments was performed by Wilcoxon-Mann-Whitney U test.

3. Results

3.1. Hyperthermia at 56 °C induces apoptosis followed by secondary necrosis in TRAMP-C2 cells in vitro

Hyperthermia treatment at 56 °C of TRAMP-C2 cells increased significantly the percentages of late apoptotic cells (Fig. 1A) and HMGB1 release (Fig. 1B) compared untreated TRAMP-C2 control cells. More precisely, the treatment induced secondary necrosis in about 90% of cell population; heating time or 37 °C incubation time extensions did not result in any further cell death increase (data not shown).

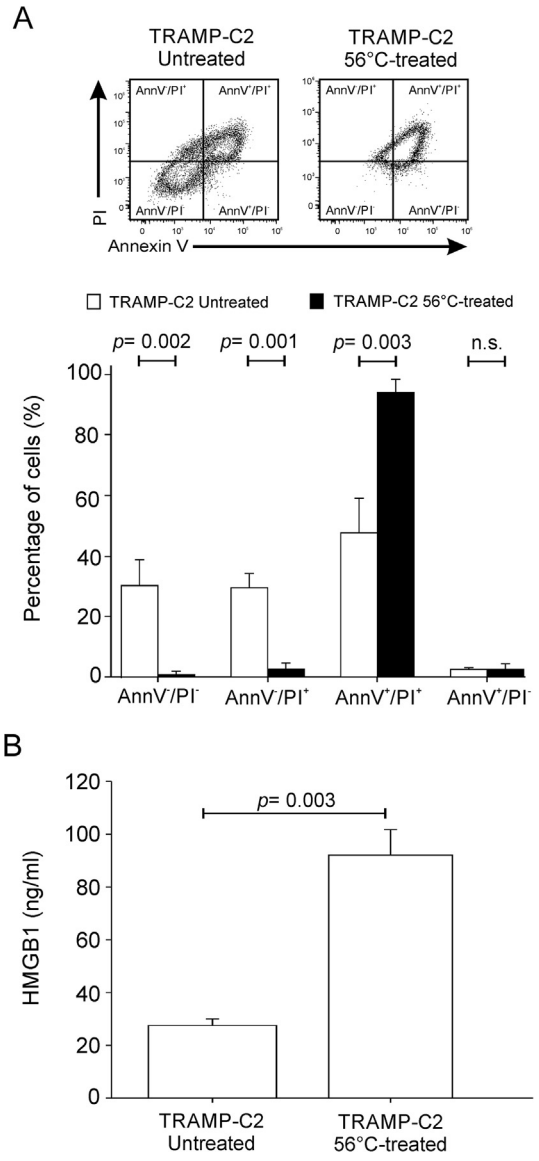

Fig. 1. Hyperthermia induces apoptosis followed by secondary necrosis and HMGB1 secretion in TRAMP-C2 cells *in vitro*. The percentages of either early apoptotic ($AnnV^+/PI^-$), primary necrotic ($AnnV^-/PI^+$) and late apoptotic (secondary necrotic) ($AnnV^+/PI^+$) cells or HMGB1 secretion were assessed in TRAMP-C2 cells subjected to 30 min of 56 °C hyperthermic treatment, followed by 4 h resting at 37 °C. (A) Annexin V/PI staining identified apoptosis induction in heat-treated cells (TRAMP-C2 56 °C-treated) compared to untreated control (TRAMP-C2 untreated). Dot plots refer to a representative experiment out of three. Histograms, mean ± SD of three independent experiments. Statistic analysis was performed by Wilcoxon-Mann-Whitney U between TRAMP-C2 cells untreated vs. 56 °C-treated TRAMP-C2 cells: $AnnV^-/PI^-$, $p = 0.002$; $AnnV^-/PI^+$, $p = 0.001$; $AnnV^+/PI^+$, $p = 0.003$; $AnnV^+/PI^-$, not statistically significant (n.s). (B) ELISA assay of HMGB-1 protein in the supernatants of untreated or 56 °C-treated TRAMP-C2 cells. Data refer to the mean ± SD of 3 independent experiments. Statistic analysis was performed by Wilcoxon-Mann-Whitney U test: TRAMP-C2 cells untreated vs. 56 °C-treated TRAMP-C2 cells, $p = 0.003$.

3.2. Hyperthermia treatment at 56 °C of TRAMP-C2 cells increases iDCs endocytosis and activation status through tumour cell-derived DAMPs release

TRAMP-C2 heated cells were then tested for their capability to be endocytosed by iDCs. iDCs were co-cultured for 24 h with PKH26-labelled hyperthermia-treated TRAMP-C2 cells and then stained with anti-CD11c. iDCs endocytosis was evaluated as double-positive ($CD11c^+PKH26^+$) cells by flow cytometry, whereas $PKH26^+$ $CD11c^-$ and $PKH26^-CD11c^+$ cell populations refer to TRAMP-C2 tumour cells and iDCs respectively. Hyperthermia treatment resulted in a statistically significant increase (about 10%) of endocytosis-engulfed TRAMP-C2 cells by iDCs compared to untreated cells (Fig. 2A); the strong $CD11b^-PKH26^+$ cell population represents TRAMP-C2 cells not engulfed by iDC. These data were confirmed by confocal laser scanning microscopy (Fig. 2B). The induction of higher endocytosis activity was coupled with

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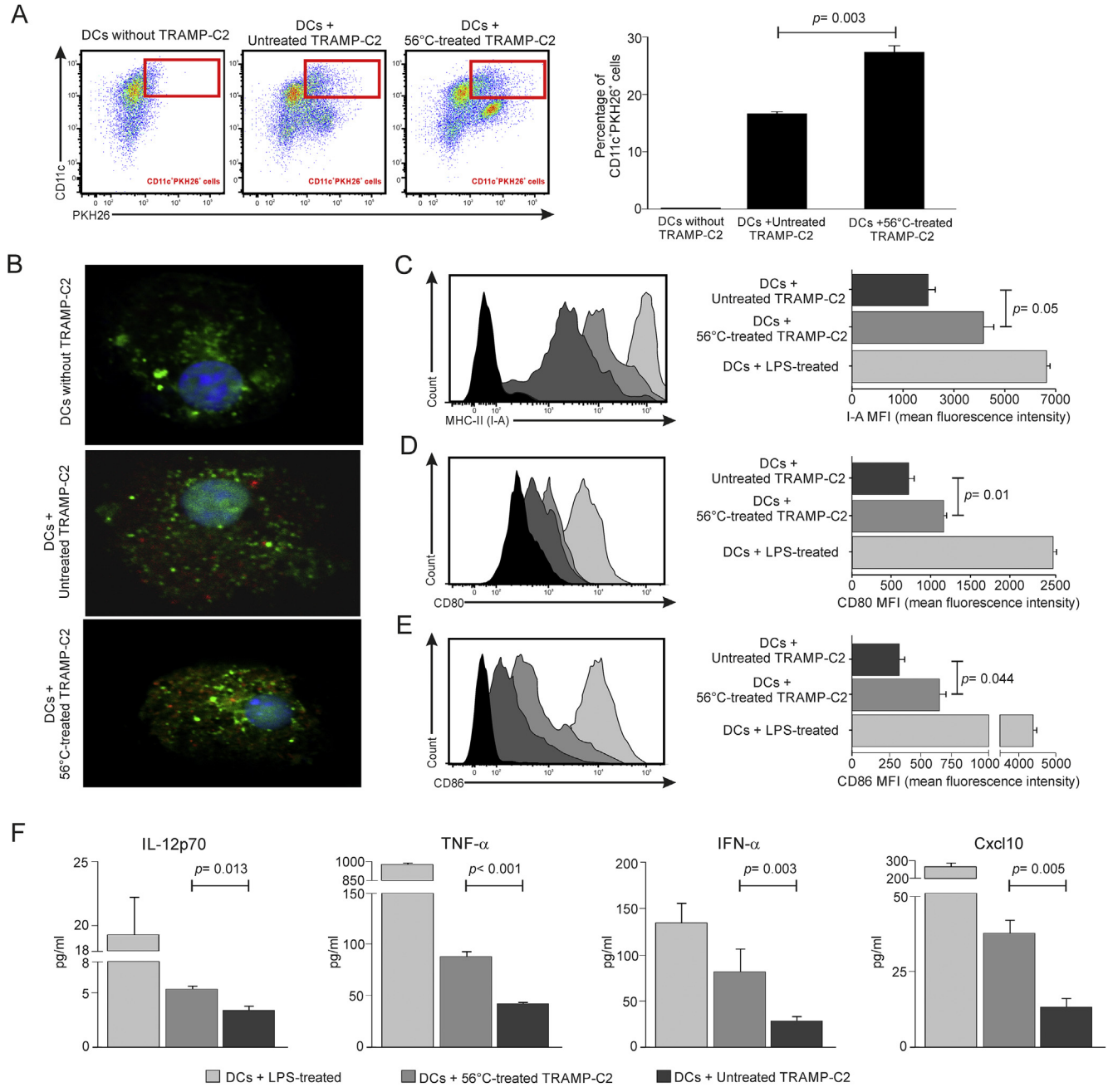

Fig. 2. Validation of iDCs maturation after 56 °C- treated TRAMP-C2 cells endocytosis. (A) Endocytosis index of untreated or 56 °C-treated TRAMP-C2 cells (identified as PKH26$^+$ cells) by DCs (identified as CD11c$^+$ cells). Dot plots refer to a representative experiment out of three. Double positive CD11c$^+$PKH26$^+$ cells were gated on singlet cells. Histograms refer to the mean ± SD of 3 independent experiments. Statistic analysis was performed by Wilcoxon-Mann-Whitney *U* test between DCs co-cultured with TRAMP-C2 cells untreated vs 56 °C-treated TRAMP-C2 cells, $p = 0.003$. (B) Immunofluorescence analysis by confocal microscopy of purified DCs by immunomagnetic sorting as CD11c$^+$ cells and labelled with FITC-CFSE probe in presence of untreated or 56 °C-treated TRAMP-C2 cells labelled with PE-PKH26 probe. Flow cytometry analysis of MHC-II I-A (C), CD80 (D) and CD86 (E) expression levels on DCs (gated as CD11c$^+$ cells) in presence of different *in vitro* stimuli: untreated TRAMP-C2 cells (dark grey area and bar); 56 °C-treated TRAMP-C2 cells (middle grey area and bar); LPS treatment as positive control (light grey area and bar). Untreated DCs were used as control (black area). Flow cytometry plots refer to one representative experiment out of five with similar outcome. Histogram plots refer to the mean ± SD of 3 independent experiments. Statistic analysis by Wilcoxon-Mann-Whitney *U* test between DCs in presence of untreated TRAMP-C2 cells or 56 °C-treated TRAMP-C2 cells: MHC-II I-A (C), $p = 0.05$; CD80 (D), $p = 0.01$ and CD86 (E), $p = 0.044$. (F) TRAMP-C2 cells were treated or not at 56 °C for 30 min, followed by incubation at 37 °C for 4 h. After that TRAMP-C2 cells were co-incubated with DCs for 24 h. LPS was used as positive control of DCs activation. The cytokines (IL-12p70, TNF-α, IFN-α and CXCL-10) released in the supernatant of these co-cultures were quantified by ELISA. Histogram data refer to the mean ± SD of 3 independent experiments. Statistical analysis was performed by Student's *t*-test.

improved iDCs maturation, as monitored by a statistically significant increase of MHC-II (I-A), CD80 and CD86 activation markers expression (Fig. 2C–E) and of IL-12p70, TNF-α, IFN-α and CXCL-10 cytokines production (Fig. 2F), linked to iDC activation [32,33].

Thus 56 °C-treated TRAMP-C2 cells improve the endocytic activity of iDCs favouring their maturation to functional mature DCs (mDCs), by secreting more DAMPs than untreated, similarly to the treatment with the pathogen associated molecular pattern

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(PAMP) LPS, one of the best known DC activators [26,27], used as a control of DC activation and maturation.

3.3. In a prophylactic immunization set up, administration of 56 °C-treated TRAMP-C2 cells to naïve mice induces protection against tumour growth of TRAMP-C2 subsequently inoculated

To test the *in vivo* vaccination efficacy of 56 °C-heated TRAMP-C2 cells, four groups of C57BL/6J naïve male mice each were immunized according to the following prophylactic schedules (*pro-1/4*):

- pro-1. : s.c. injection of mDCs *in vitro*-loaded with 56 °C treated TRAMP-C2 cells;
- pro-2. : s.c. injection of 56 °C treated TRAMP-C2 cells without washing out supernatant;
- pro-3. : s.c. injection of 56 °C treated TRAMP-C2 cells washed out of supernatant and resuspended in PBS;
- pro-4. : unvaccinated controls.

Three weeks after immunization, each group of mice was challenged by s.c. injection with TRAMP-C2, either IFN-γ-treated or untreated.

Tumour growth was monitored twice a week until 60 days from challenge, which was chosen as experimental endpoint. Statistical analysis comparing the different experimental conditions was always performed along the whole time of 60 days from challenge. We observed an increase in the number of mice that did not develop tumours (*event-free survival* = EFS) and in the number of either tumour-free mice or tumour-bearing mice showing a delay in tumour growth (*overall survival* = OS), in almost all mice immunized with *pro-1* or *pro-2* compared to mice treated *with pro-4* or *pro-3*.

Among mice challenged with IFN-γ-untreated TRAMP-C2 cells (Fig. 3A and B) we detected a statistically significant increase of OS only comparing mice immunized with *pro-1* to *pro-4* (Fig. 3A) and of EFS comparing mice immunized with *pro-1* to *pro-4* and *pro-3* (Fig. 3B).

Mice challenged with IFN-γ-TRAMP-C2 cells showed a statistically significant increase of: both OS (Fig. 3C) and EFS (Fig. 3D) comparing mice immunized with *pro-1* to mice immunized with *pro-3* or to *pro-4*; EFS only when comparing mice immunized with *pro-2* to *pro-1* and to *pro-3* (Fig. 3D). Thus, the prophylactic vaccination outcome was better appreciated when challenging with IFN-γ-treated TRAMP-C2 cells.

Among the different prophylactic vaccination schedules, *pro-1* resulted the most efficient; *pro-2* supported tumour protection as well, but at lower levels. We can speculate that 56 °C-treated

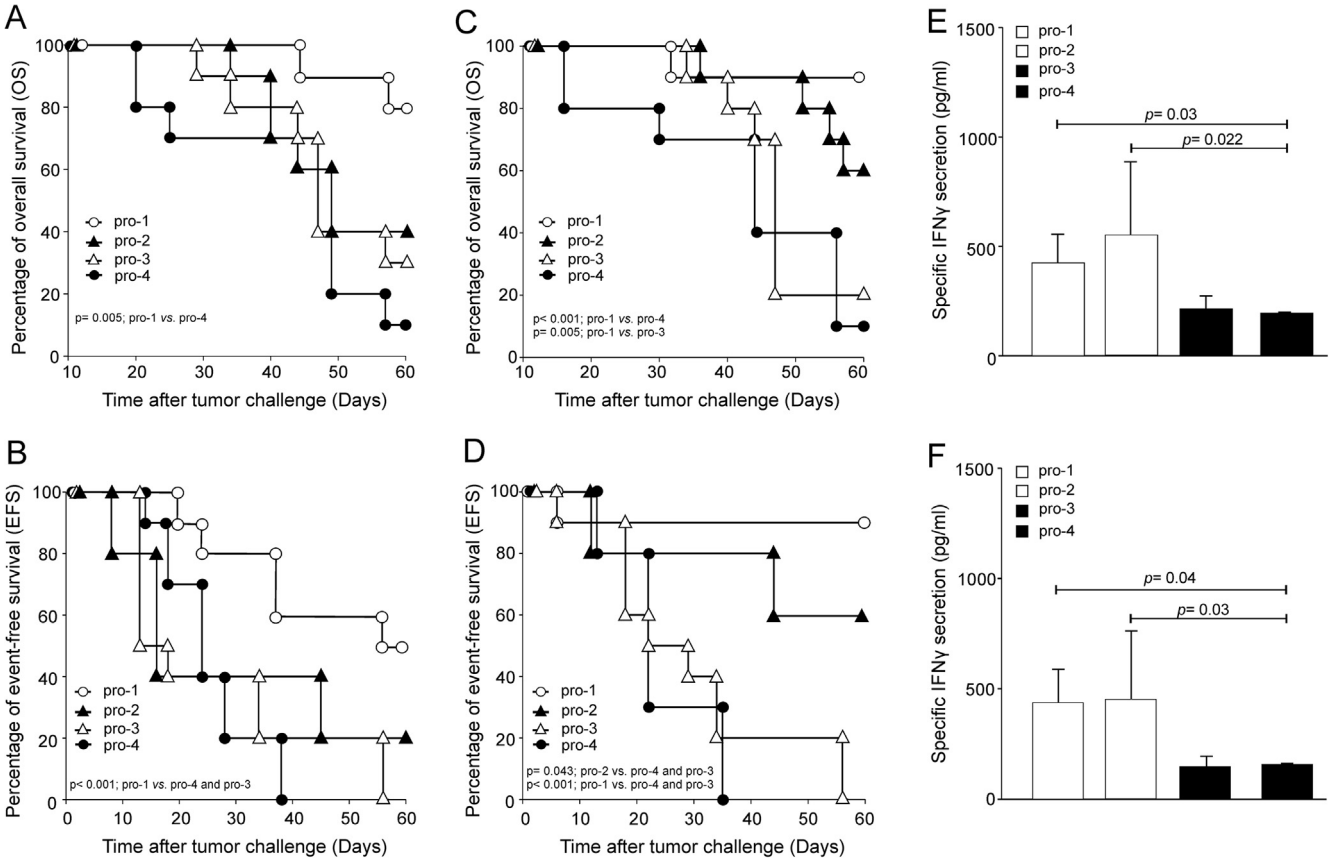

Fig. 3. Effects of prophylactic vaccination with 56 °C-treated TRAMP-C2 cells on IFN-γ-treated or -untreated TRAMP-C2 tumour growth. C57BL/6J mice were either *unvaccinated (pro-4*, n = 20; black circle) or immunized with: DCs loaded with 56 °C-treated TRAMP-C2 cells *(pro-1*, n = 20*;* white circle), 56 °C-treated TRAMP-C2 cells with 56 °C supernatant *(pro-2*, n = 20*;* black triangle) or TRAMP-C2 56 °C without supernatant *(pro-3*, n = 20*;* white triangle). After three weeks from immunization, mice were challenged with IFN-γ-treated (n = 10) or untreated TRAMP-C2 cells (n = 10). (A) Overall survival (OS) of mice challenged by s.c. injection with TRAMP-C2 cells. Statistic analysis by Kaplan Meier log rank test: *pro-1* vs. *pro-4; p* = 0.005. (B) Event free survival (EFS) of mice challenged by s.c. injection with TRAMP-C2 cells. Statistic analysis by Kaplan Meier log rank test: *pro-1* vs *pro-4 and pro-3; p* < 0.001. (C) OS of mice challenged by s.c. injection with IFN-γ-treated TRAMP-C2 cells. Statistic analysis by Kaplan Meier log rank test: *pro-1* vs *pro-4, p* < 0.001; *pro-1* vs *pro-3, p* = 0.005. (D) EFS of mice challenged by s.c. injection with IFN-γ-treated TRAMP-C2 cells. Statistic analysis by Kaplan Meier log rank test: *pro-1* vs *pro-4 and pro-3, p* < 0.001; *pro-2* vs *pro-4 and pro-3, p* = 0.043. (E, F) IFN-γ ELISA of supernatants derived from a five-day co-culture of γ-irradiated untreated (E) or IFN-γ-treated (F) TRAMP-C2 cells in presence of splenocytes derived from unvaccinated mice (*pro-4,* black bar), or mice immunized with *pro-1* (white bar), *pro-2* (light grey bar) or *pro-3* (dark grey bar). Statistic analysis was performed by Wilcoxon-Mann-Whitney *U* test: *pro-1* vs *pro-4, p* = 0.03 and *pro-2* vs. *pro-4, p* = 0.022 for panel E; *pro-1* vs *pro-4, p* = 0.04 and *pro-2* vs *pro-4, p* = 0.03 for panel F.

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TRAMP-C2 cells with DAMP-containing sup *(pro-2)* delivered *in vivo* did not activate resident DCs with the same therapeutic efficiency that is achieved *in vitro*. If 56 °C-treated TRAMP-C2 cells were washed out of the supernatant before injection *(pro-3)*, tumour protection was lost, confirming the critical role in DCs activation played by tumour-derived DAMP release following hyperthermia treatment [16].

To confirm the triggering of a TRAMP-C2-specific T cell response, the supernatants of co-cultures of spleen cells derived from vaccinated *(pro-1/3)* and unvaccinated mice with either TRAMP-C2 cells (Fig. 3E) or TRAMP-C2 cells pre-treated with IFN-γ (Fig. 3F), were tested by IFN-γ ELISA. Tumour-spleen cells co-cultures from mice immunized with either *pro-1* or *pro-2* compared to cultures of spleen cells from both mice immunized with *pro-3* or *pro-4*, showed a statistically significant increase of IFN-γ production (Fig. 3E and F). Immunization with *pro-1* and *pro-2* induced a similar specific T cell response against tumour, suggesting that in *pro-2*, 56 °C-treated TRAMP-C2 cells are endocytosed by resident iDCs, favouring their maturation. This was not the case of *pro-3*, highlighting the functional role of tumour-derived DAMPs in triggering a tumour-specific cytotoxic immune response.

3.4. In a therapeutic immunization set up, administration of mDCs loaded with 56 °C-treated TRAMP-C2 cells to tumour-bearing mice induces protection against TRAMP-C2 tumour growth only when combined with chemotherapy

Given the promising results achieved immunizing with mDCs *in vitro*-loaded with 56 °C-treated TRAMP-C2 in the prophylactic set up (Fig. 3), we explored the feasibility of this approach in a therapeutic regimen, either alone or in association with docetaxel chemotherapy [1] (Fig. 4).

Naïve mice were injected s.c. with 5×10^6 tumorigenic TRAMP-C2 cells. Thirteen days later, they were divided in four groups according to therapeutic immunization schedule (*ther-1/4*) as follows:

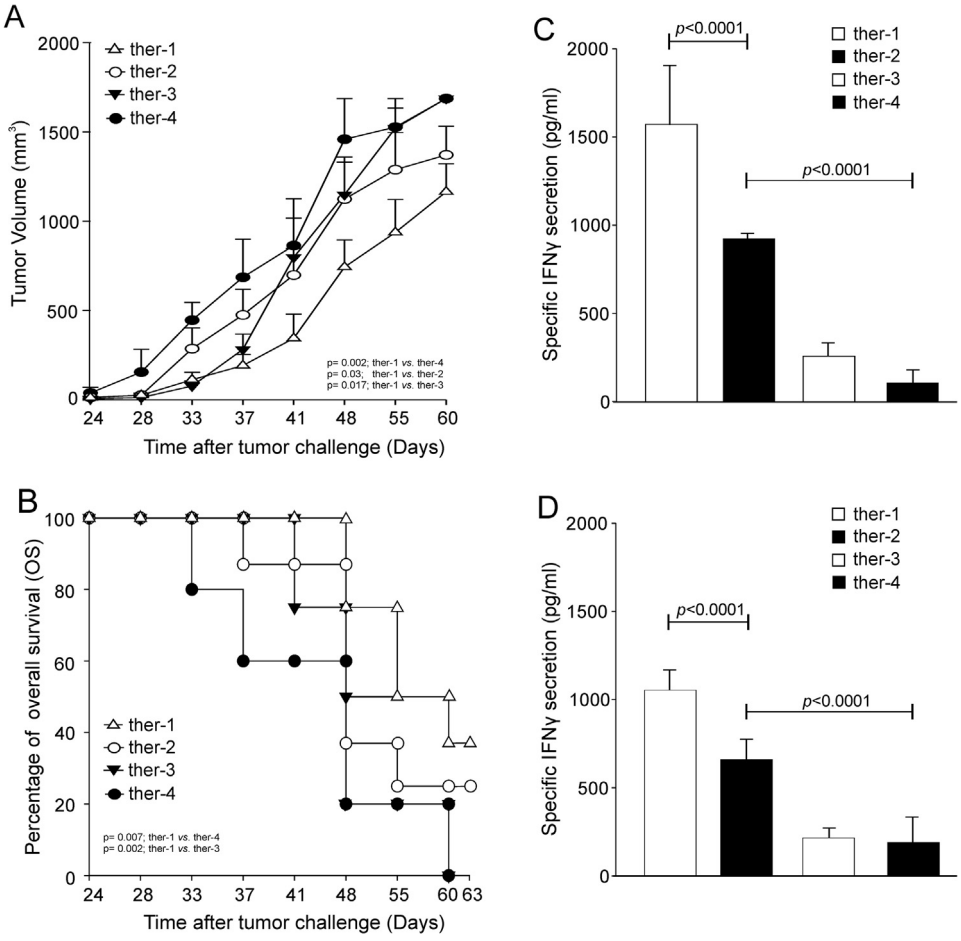

Fig. 4. Efficacy of therapeutic vaccination with mDCs loaded with 56 °C-treated TRAMP-C2- associated to chemotherapy on TRAMP-C2 tumour growth. Naïve mice (n = 54) were injected s.c. with 5×10^6 TRAMP-C2 cells. Thirteen days later, tumour-bearing mice were treated according to the following schedules: *ther-1*, combination of docetaxel and vaccination with 56 °C-treated-TRAMP-C2 *in vitro*-loaded mDCs (*n = 13*, white triangle); *ther-2*, vaccination treatment with 56 °C-treated-TRAMP-C2 *in vitro*-loaded mDCs (*n = 13*, white circle); *ther-3*, docetaxel treatment (*n = 13*, black triangle) and *ther-4*, untreated control mice (*n = 15*, black circle). A) Tumour growth of treated mice was reported as tumour volume variations over the time. Statistic analysis was performed using χ^2 test with Yates correction: *ther-1* vs *ther-4*, $p = 0.002$; *ther-1* vs *ther-2*, $p = 0.03$ and *ther-1* vs *ther-3*, $p = 0.017$. (B) Overall survival (%) of treated mice. Statistic analysis by Kaplan Meier log rank test: *ther-1* vs *ther-4*, $p = 0.007$ and *ther-1* vs *ther-3*, $p = 0.002$. (C) and (D) IFN-γ ELISA of supernatants derived from a five-day co-culture of γ-irradiated untreated (C) or IFN-γ-treated (D) TRAMP-C2 cells in presence of splenocytes isolated from treated mice. Statistic analysis was performed by Wilcoxon-Mann-Whitney *U* test: *ther-2* vs *ther-4*, $p \leq 0.0001$ and *ther-2* vs *ther-1*, $p \leq 0.0001$ for panel C; *ther-2* vs *ther-4*, $p \leq 0.0001$ and *ther-2* vs *ther-1*, $p \leq 0.0001$ for panel E.

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ther-1. : combination of docetaxel preconditioning, followed two days later by vaccination with mDCs *in vitro*-loaded with 56 °C-treated-TRAMP-C2 delivered s.c.;
ther-2. : vaccination treatment only, with mDCs *in vitro*-loaded with 56 °C-treated-TRAMP-C2 delivered s.c.;
ther-3. : docetaxel treatment only;
ther-4. : control mice unvaccinated and docetaxel-untreated.

Only the combination of docetaxel and vaccination treatments induced a statistically significant reduction of tumour growth rates (Fig. 4A) and increased overall survival, compared to the other treatments (Fig. 4B).

To test the presence of a TRAMP-C2-specific T cell response, spleen cells derived from 5 mice from each treatment group were co-cultured with IFN-γ-untreated and IFN-γ-treated TRAMP-C2 cell targets, and supernatants were analysed for the presence of IFN-γ as activation index of spleen-derived T cells, in ELISA assays (Fig. 4C and D). A statistically significant increase of IFN-γ production was found comparing *ther-4* to *ther-2*, *ther-4* to *ther-1* and *ther-2* to *ther-1*, against both IFN-γ-conditioned and IFN-γ-unconditioned TRAMP-C2 targets.

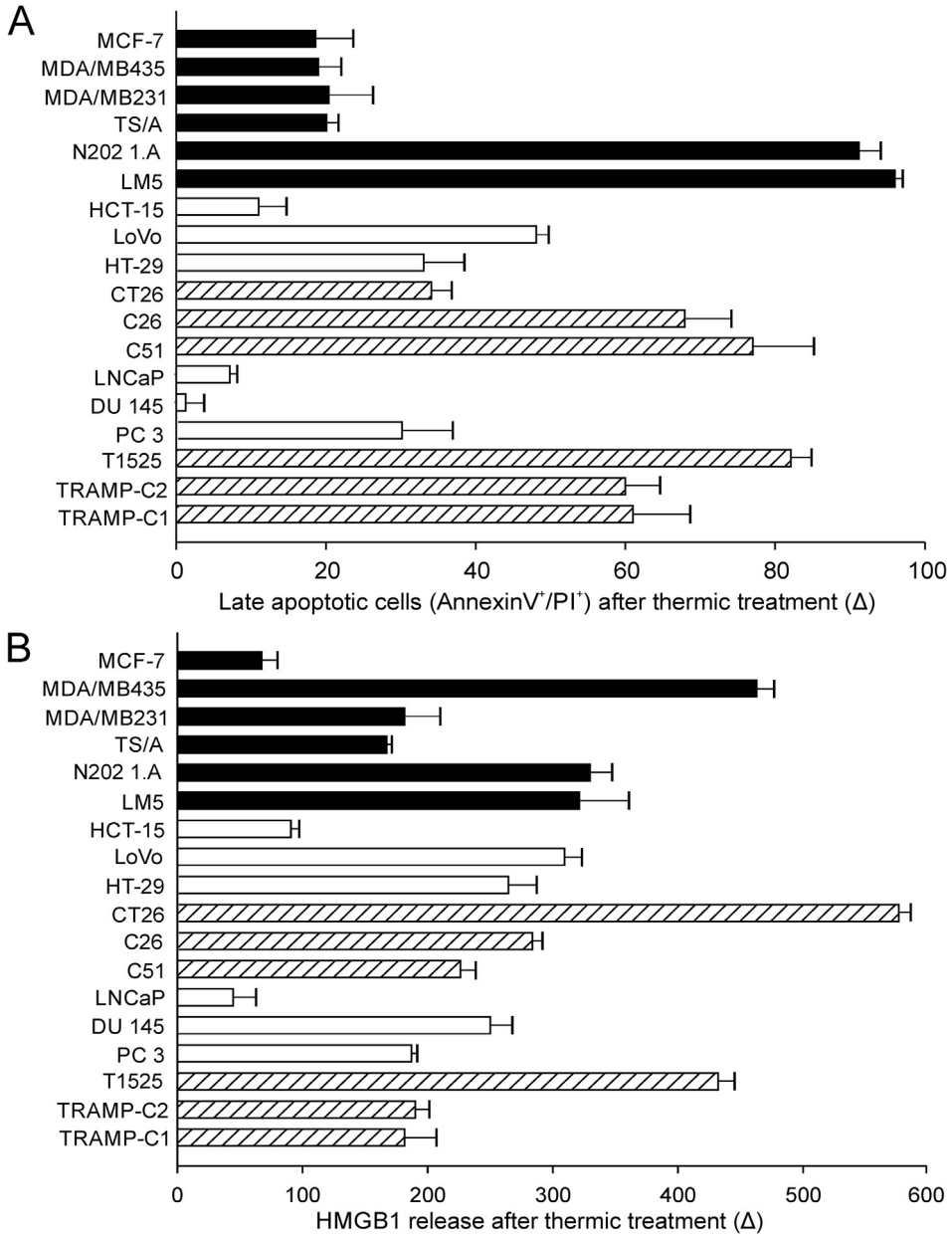

Fig. 5. Quantification of apoptosis/secondary necrosis and HMGB1 release in different human and murine tumour cell lines following 56 °C hyperthermia treatment. Human and murine tumour cell lines of different histologic types were treated at 56 °C and the levels of late apoptosis/secondary necrosis induction and HMGB1 release in the supernatant were compared. Histograms colour: dark grey for human mammary adenocarcinoma; dark grey dashed for mouse mammary adenocarcinoma; light grey for human colon carcinoma; light grey dashed for mouse colon carcinoma; white for human prostate cancer; white dashed for mouse prostate cancer. Histogram data refer to the mean ± SD of 3 independent experiments. (A) Fold increase (Δ) of late apoptotic cells (AnnexinV$^+$/PI$^+$) after 56 °C hyperthermic treatment, compared to untreated. (B) Fold increase (Δ) of HMGB1 release by late apoptotic cells after 56 °C hyperthermic treatment, compared to untreated.

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Thus, the vaccination regimen alone or in association with chemotherapy promoted a TRAMP-C2-specific immune response, which in the absence of chemotherapy was not able to contrast tumour growth efficiently. Finally, the docetaxel treatment alone did not elicit any TRAMP-C2-specific immune response, as the IFN-γ secretion levels were comparable to those of untreated animals.

3.5. Hyperthermia at 56 °C induces apoptosis followed by secondary necrosis in human and mouse cancer cell lines derived from different tissues in vitro

The 56 °C hyperthermia protocol used to treat TRAMP-C2 cells *in vitro* was applied to evaluate the induction of late apoptosis and of HMGB1 release in several human and mouse tumour cell lines of different histologic origin (Fig. 5). In general, the percentage of late apoptotic cells increased after thermic treatment (Fig. 5A). Conversely, the levels of secreted HMGB1 in the supernatant of heat-treated cell lines increased with respect to the untreated control in all the tested tumour cell lines, at different levels (Fig. 5B). Therefore, hyperthermia at 56 °C as immunotherapeutic approach, appears to be eligible to treat a large spectrum of tumours with different histology.

4. Discussion

Our data demonstrate that triggering a tumour-protective immune response using an immunotherapeutic strategy based on 56 °C-hyperthermia-treated TRAMP-C2 tumour cell vaccine is feasible. This approach appears more translatable to clinical protocols than genetically modified tumour cells, recently explored in the same model [19].

56 °C-treated TRAMP-C2 vaccine induced an *in vivo* protection, provided that the DAMPs-containing-56 °C-incubating supernatant was present in the immunizing inoculum, although with a lower impact than immunization with mDCs *in vitro*-loaded with 56 °C-treated TRAMP-C2 cells. A reasonable explanation is that not all the material can be sensed and up-taken by resident DCs *in vivo* as it may happen *in vitro*. According to that, immunization with 56 °C-treated tumour cells washed after heating did not protect against tumour growth, because host DCs were not activated in the absence of DAMPs.

IFN-γ treatment prior tumour challenge rendered tumour cells more susceptible to CTL killing, through the up-regulation of antigen processing machinery (APM) TAP-1/TAP-2 and LMP-2/LMP-7 components, associated with an increase of cell-surface expression of MHC-I/peptide complexes [19]. Accordingly, down-regulation of MHC-I expression is a frequent feature of PCa [34,35], sometimes controversial [36]. As some human prostate carcinoma cell lines, TRAMP-C2 shows a down-regulated MHC-I expression [37,38], due to the inhibitory effect of tumour-secreted TGF-β, counter-regulated by the IFN-γ-treatment [19].

To evaluate the translatability of this vaccination approach to clinics, we switched to a therapeutic vaccination set up. DCs loaded with 56 °C-treated TRAMP-C2 vaccine was tested alone or associated to docetaxel chemotherapy. The overall survival dropped down compared to prophylactic immunization, although the results obtained with the combined treatment docetaxel/immunization showed a statistically significant reduction of both tumour growth rate and increase of overall survival, compared to single treatments.

In the TRAMP-C2 system, this 56 °C hyperthermia protocol appeared to work better than high hydrostatic pressure, that raised a specific $CD8^+$ T cell response without decreasing tumour growth rates, and when combined with docetaxel produced similar results, although with three deliveries administered at alternate weeks, whilst our results were obtained with one single delivery of 56 °C-treated-TRAMP-C2 combined with docetaxel [39].

In addition to its cytotoxic effects on PCa [1], docetaxel induces immunogenic modulation by increasing TAP-2 and calnexin APM molecules expression [40]. Thus, in the TRAMP-C2 tumour model, similarly to what we previously described for IFN-γ [19], docetaxel creates synergy with the 56 °C-treated-TRAMP-C2 therapeutic immunization regimen, resulting in a tumour-protective effect able to delay tumour growth in a statistically significant manner. These results have potential clinical implications, strengthening the importance of combination protocols of immunotherapy and chemotherapy, and in the context of PCa, the synergic role of hyperthermia and docetaxel in the enhancement of specific anti-tumour immune response.

5. Conclusions

The results presented here strongly favour the translatability of the 56 °C hyperthermia treatment to clinics in association to docetaxel, by modulating the thermal energy applied and the time of application in HIFU, RFA, IRE or MWA treatments, in order to extend the transitional zone of sub-lethal hyperthermia at temperatures close to 56 °C, to increase the release of DAMP molecules and pro-inflammatory cytokines from tumour cells.

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Disclosures

There is not conflict of interest to disclose.

Author contributions

M Mazzocco and M Martini and performed the initial experiments of prophylactic immunization and contributed to the writing of the first part of the article. S Sandri, FDS, AF and RT performed the final experiments of therapeutic immunization and contributed to the writing of the second part of the article. SG and DB contributed to the design of the study and to the discussion of the results. S Sartoris and SU designed the study and wrote the article.

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