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Regulation of HSP70 on activating macrophages using PDT-induced apoptotic cells

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Although anti-tumor immunological responses have been mainly associated with necrosis, apoptosis-associated immune responses have been recently suggested as well. In this study, we investigated anti-tumor immune responses and regulatory mechanisms of HSP70 using apoptotic cells induced by photodynamic therapy (PDT). The relationships between HSP70 release, HSP70 translocation and macrophage responses were studied using confocal fluorescence microscopy, FACS and ELISA. Macrophages incubated with apoptotic cells as well as necrotic tumor cells showed a high level of TNFα secretion. Apoptotic cells but not the apoptotic cell supernatants induced TNFα secretion. During both necrosis and apoptosis processes, the TNFα production was diminished drastically when HSP70 or TLR-2 was inhibited. After the PDT treatment, cytoplasmic HSP70 was released from the necrotic cells, while HSP70 rapidly translocated to the surface of the apoptotic cells. Furthermore, the TNFα secretion and the tumor cytotoxicity of splenocytes from mice immunized with apoptotic cells appeared similar to that of splenocytes immunized with necrotic cells. Our *in vitro* and *in vivo* results show that apoptosis can potentially have higher impact in inducing immunological responses, hence clarifying the immunological regulatory mechanisms of HSP70 under cell apoptosis and necrosis induced by PDT treatment. These findings could lead to an optimal PDT treatment based on immunological responses.

Key words: PDT; HSP70; apoptotic cells; immunological regulation; macrophage

The majority of cytotoxic therapies results in apoptosis of the target cells.[1,2] Cells that are replaced daily through normal apoptotic processes do not routinely induce autoimmunity. However, whether treatment-induced apoptosis could cause immune response is still being debated. In some cases, apoptosis has been shown to be immunologically "silent" and potentially anti-inflammatory.[3,4] In other cases, it is believed that apoptosis can also be an effective vaccine modality for activation of antigen-specific immune responses.[5–7]

To induce an anti-tumor immune response, cell killing must be sensed and distinguished from normal cell death processes. However, most cancer therapies lack such a distinction. Photodynamic therapy (PDT) has been shown to induce certain immunological reactions.[8–12] It produces photooxidative lesions, which directly cause photodamage of proteins, lipids and other molecules at the sites where the photosensitizer accumulates, leading to tumor cell death either by apoptosis or necrosis.[13–16] It has been proposed that photooxidative lesions produced in PDT-treated tumors are recognized by the host as the "altered self," prompting a strong acute inflammatory reaction and other immune responses, including the development of adaptive immunity against the treated cancerous lesion, which contributes to the therapeutic outcome of PDT.[8,12] However, whether and how PDT-induced apoptotic cells could cause immune response against tumors are not well known.

Macrophages play an important role in the clearance of dying and dead cells as one of the professional antigen-presenting cell (APCs).[17–19] They can both sense and respond to different mechanisms of cell death both *in vitro* and *in vivo*. There is an evidence that macrophages can act as both positive and negative regulators of immune responses to tumors.[17,18] However, it is not clear what the necessary danger signals are that positively stimulate macrophages. Accumulating evidence indicates that HSP70 plays the role of maturation stimulation,[20–22] by chaperoning antigenic peptides and channeling them into APCs.

Heat shock proteins (HSPs) perform important cellular protective functions in folding and intracellular transport of newly synthesized proteins, preventing protein aggregation and misfolding.[23,24] In addition, these molecules also participate in signal transduction pathway[25,26] and regulate inflammatory and immune responses.[27,28] HSPs have been assumed to inhabit in nearly all intracellular compartments; it has been recently shown to be expressed on outer cellular membrane and even released from damaged and viable cells.[21,29,30]

Combing the 2 facts that HSP70 regulates immune response and PDT induces cell surface expression of HSP70 during apoptosis, we speculate that PDT-induced apoptosis could trigger immune response because of the HSP70 expression on the cell surface. We further investigate the immunological effects of PDT-induced apoptosis *in vitro* and *in vivo*, as well as the regulatory mechanisms of HSP70 in the induced immune responses.

Material and methods

Chemicals and plasmids

Cholera toxin B subunit (CTxB)-Alexa 594 (0.5 μg/ml, Molecular Probes, Inc., USA), a fluorophore probe, was used to stain macrophages, Hoechst/PI (Sigma, St. Louis, MO) were used to label nuclear DNA, Cell Tracker Green (Molecular Probes) was used to label cells. The optimal concentrations and incubation times for the probes were determined experimentally.

The following chemicals and commercially available assay kits were used: Photofrin (Sinclair Pharmaceuticals, GU7 2AB, UK), rabbit anti-mouse HSP70, rat anti-mouse TLR2 or rat anti-mouse TLR4 (all blocking antibodies) (10 μg/ml, Bios, China), rat anti-human monoclonal antibody (Cell Signaling Technology, MA), apoptosis detection kit Annexin-V-FITC/PI (R&D Systems, Minneapolis, MN), TNFα and HSP70 ELISA Kit (R&D Systems).

In addition, we used Lipofectin reagent (Invitrogen Life Technologies, Inc., USA) to transfect YFP-HSP70 into target cells. YFP-HSP70 is a gift from Dr. Richard I. Morimoto of Northwestern University.

Cell culture and transfection

Murine mammary tumor line C127 cells and murine macrophage line RAW264.7 cells were used for the experiments. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 15% fetal calf serum (FCS),

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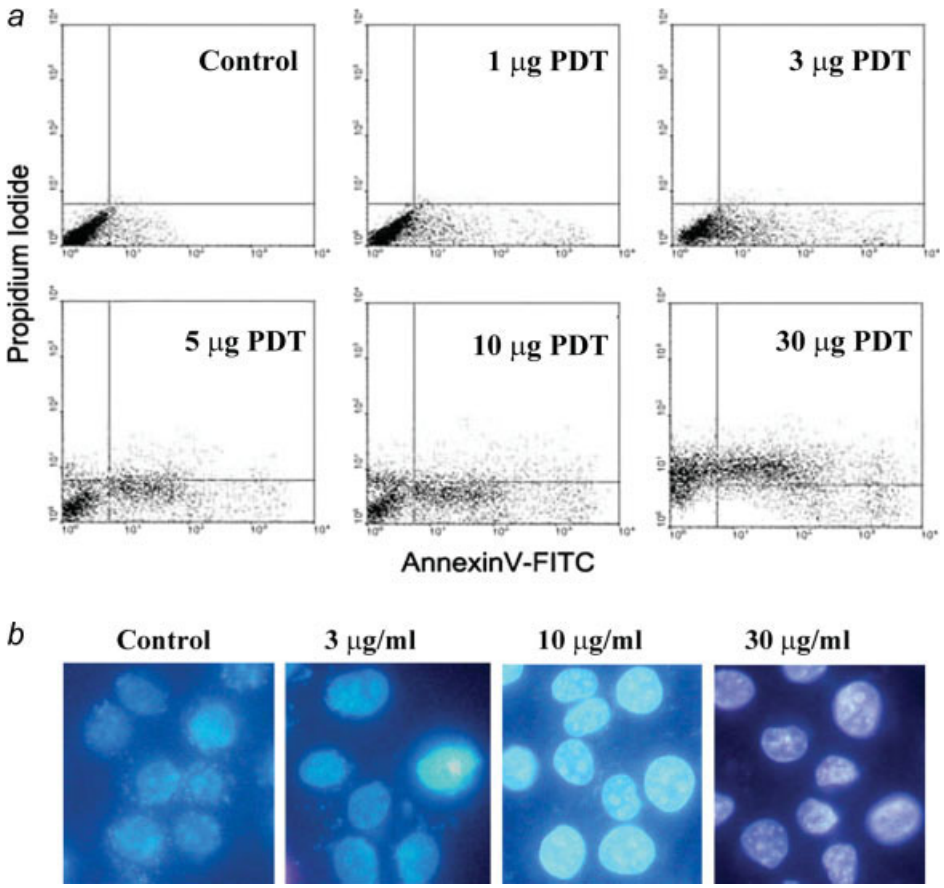

FIGURE 1 – (*a*) FACS analysis for cell death. Cells (10,000/experiment) were stained with Annexin V-FITC/PI for flow cytometry analysis after different treatments: 5 hr after PDT treatment. $n = 5$. (*b*) Cell death revealed by staining with Hoechst 33258/PI dyes in cells under different treatments, with cells of no treatment as control. Hoechst 33258 was indicated by blue and the pink represented double staining by Hoechst and PI dyes. (*c*,*d*) Time-lapse microscopy of cell death. Optical and fluorescent images (annexin V and PI), and quantified fluorescent emission (normalized to the maximum emission) of PDT (5 J/cm^2; 10 mW/cm^2) treated cells under different Photofrin doses: 10 μg/ml (*c*) and 30 μg/ml (*d*). Before treatment, cells were stained with Annexin V-FITC and PI, and observed with confocal microscopy after treatment. Bars, SD ($n = 4$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

penicillin (100 units/ml) and streptomycin (100 μg/ml) in 5% CO_2, 95% air at 37°C in humidified incubator.

For isolation of mouse peritoneal macrophages, 8 to 10-wk-old female BALB/c mice were sacrificed and 5–10 ml Ringer solution containing 5% FCS was injected into the peritoneum. Cells were recovered, pooled, seeded in full RPMI medium further supplemented with 1 × nonessential amino acids, 10 μg/ml sodium pyruvate and 10 mM HEPES buffer solution, and left to adhere on culture dishes for 2 hr. Then medium was changed and treatments were performed.

Photodynamic therapy of tumor cells

For photodynamic therapy, cells growing in 35 mm Petri dishes were incubated in the dark with Photofrin (0–30 μg/ml in complete growth medium) for 20 hr, rinsed with PBS, and exposed to light with doses of 5 J/cm^2 (10 mW/cm^2). The light source was a He–Ne laser (HN-1000, Guangzhou, China; 632.8 nm). For the control group, cells were incubated in the same medium without treatment.

Confirmation of cell death

For confocal microscopy analysis, before PDT treatment, cells were cultured with 50 ng/ml PI and 2 μg/ml Annexin-V-FITC. For FACS analysis, Annexin-V-FITC conjugate and binding buffer were used as standard reagents. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) with an excitation at 488 nm. Fluorescent emission of FITC was measured at 515–545 nm and that of DNA-PI complexes at 565–606 nm. Cell debris was excluded from analysis by an appropriate forward light scatter threshold setting. Compensation was used wherever necessary. To assess the changes in nuclear morphology of apoptosis, cells were cultured in 35 mm glass-bottomed dishes. Three hours after PDT treatment under different doses, the cells were stained with Hoechst 33258 for 10 min at room temperature and then stained with PI at 4°C, and washed twice with PBS. The cell samples were visualized under a Nikon fluorescent microscope (mercury lamp, Ex. 330–380 nm, Em. BA 435 nm).

Confocal laser scanning microscopy

Annexin-V-FITC, PI, YFP and CTxB Red fluorescence were monitored confocally using a commercial laser scanning microscope (LSM 510/ConfoCor 2) combination system (Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40×/1.3 NA Oil DIC objective. Excitation wavelength and detection filter settings for each of the fluorescent indicators were as follows. Annexin-V-FITC fluorescence was excited at 488 nm with an Ar-Ion laser and emission was recorded through a 500–550 nm IR band-pass filter. PI fluorescence was excited at 488 nm with an Ar-Ion laser and emission was recorded through a 565–615 nm IR band-pass filter. YFP fluorescence was excited at 514 nm with an Ar-Ion laser and emission was recorded through a 530–550 nm IR band-pass filter.

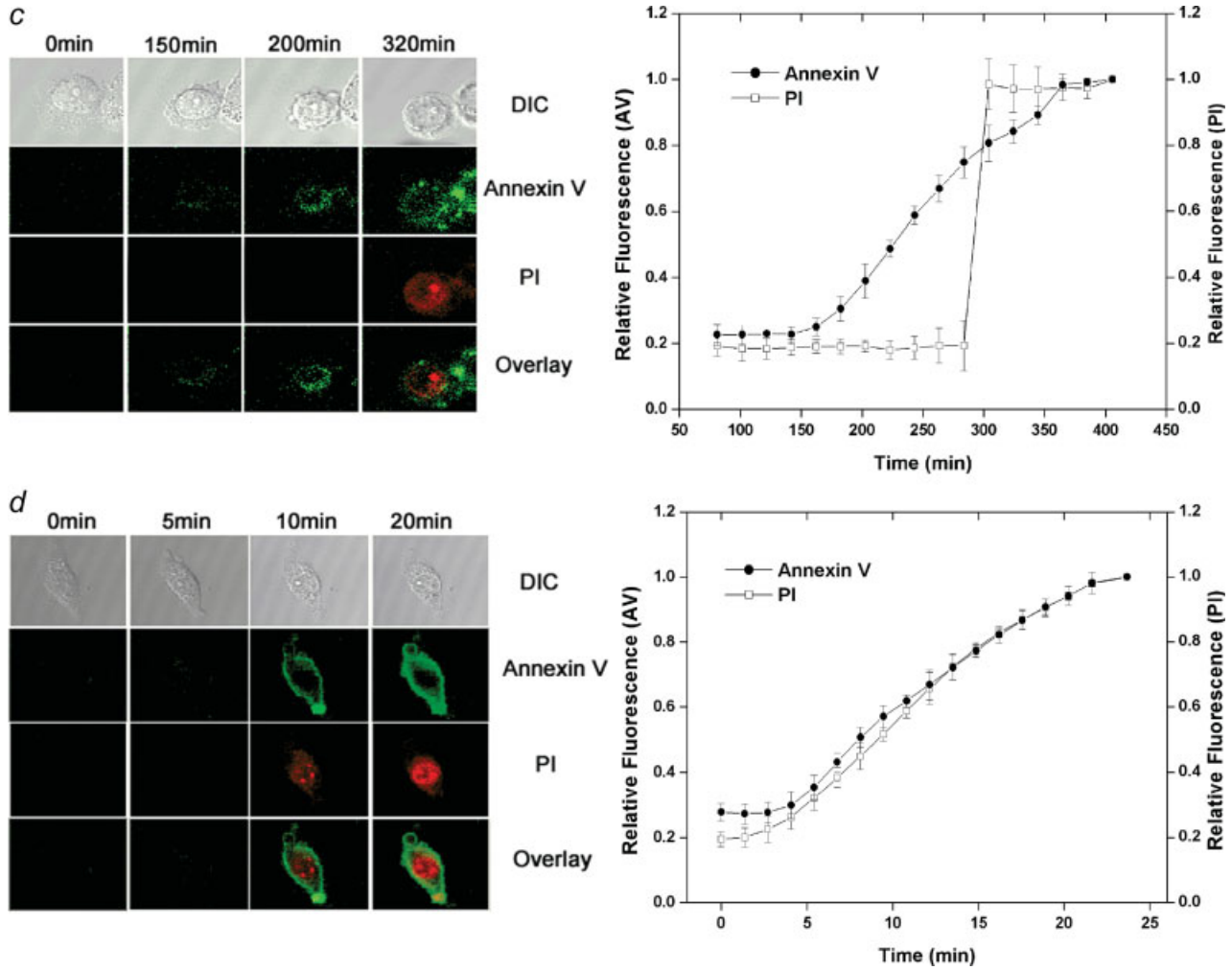

FIGURE 1 – CONTINUED.

CTxB Red fluorescence was excited at 543 nm with a He–Ne laser and emitted light was recorded through a 650 nm long-pass filter. For intracellular measurements, the desired measurement position was chosen in the LSM image. To quantify the results, the emission intensities were processed with Zeiss Rel3.2 image processing software (Zeiss, Jena, Germany).

Phagocytosis assay

To detect phagocytosis of tumor cells by macrophages in real time, laser scanning microscope was used. Before detection, RAW264.7 cells were incubated with CTxB for 30 min at 4°C in serum-free DMEM, and rinsed 3 times. The CTxB stained macrophages were incubated with PDT-treated C127 cells expressing YFP-HSP70. For FACS analysis, CTxB stained macrophages were incubated with Cell Tracker Green stained, PDT-treated C127 cells for 24 hr. The populations were measured for fluorescence by FACS analysis. The macrophages incubated with C127 cells without PDT treatment were used as controls.

Detection of cytokine and HSP70

To detect TNFα production of macrophages, macrophages were incubated with treated C127 cells in 24-well tissue culture plates. After 24 hr of co-incubation, the supernatants were collected and divided into different groups for ELISA detection.

To detect HSP70 release, the supernatants of PDT-treated C127 cell cultures were collected at different post-treatment times for ELISA analysis.

Western blot analysis

Cells were harvested in 300 μl of lysis buffer [20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerolphosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM Na3VO4 and 5 mM NaF]. The resulting lysates were resolved on 4–12% SDS-PAGE Bis-Tris gels (30 mg/lane; Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk and then probed with different antibodies. Proteins were detected by using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

In vivo *cytotoxicity assay*

Female BALB/c mice, age 6–10 weeks, were used for the experiments. The mice were immunized with treated tumor cells. Approximately 100,000 treated tumor cells in a volume of 0.5 ml were injected subcutaneously on the back of the animals. Five days after immunization, mouse splenocytes were harvested and co-cultured with mitomycin-C-treated C127 cells. TNFα secretion

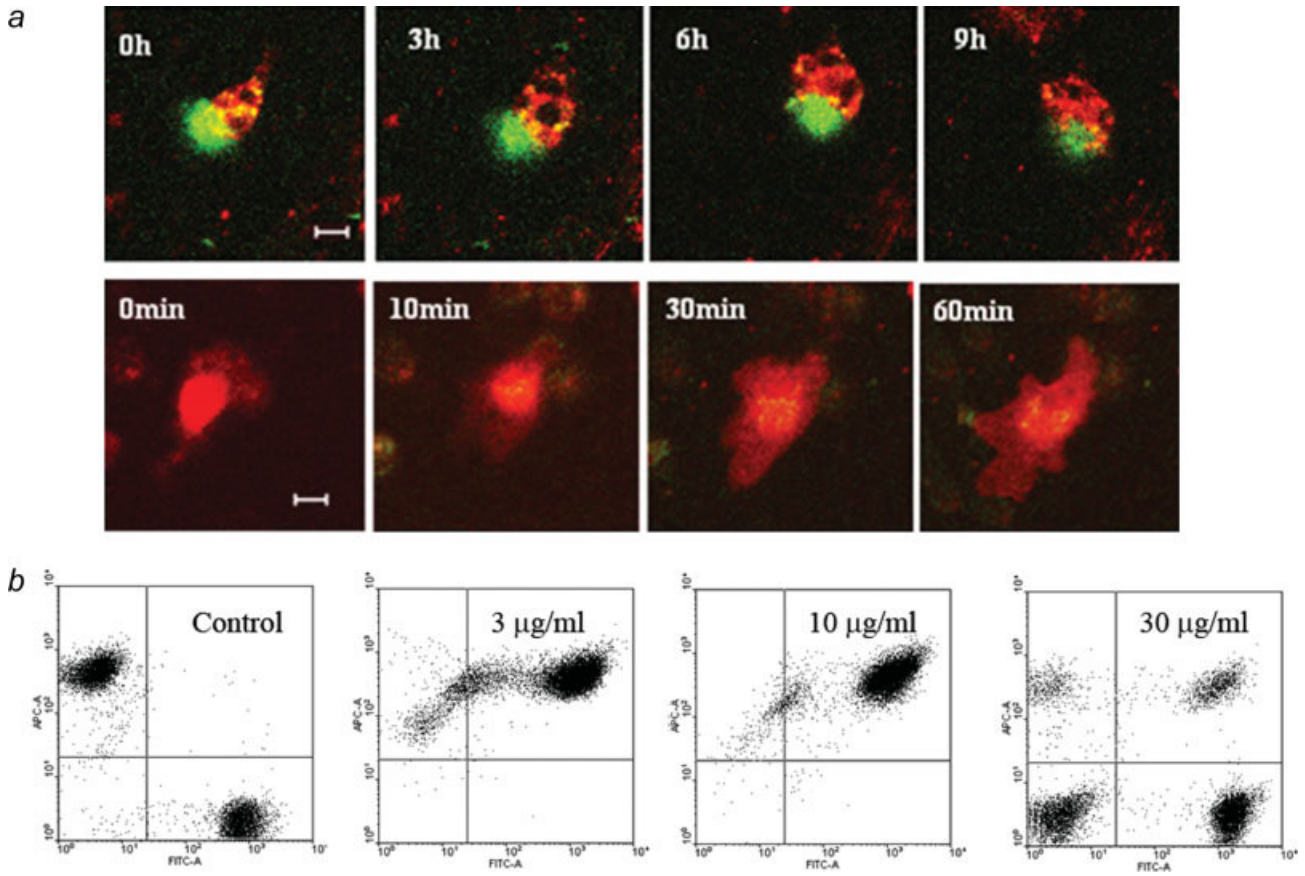

FIGURE 2 – Captures of PDT-treated cells by immature macrophages using fluorescent imaging and FACS analysis. (*a*) Captures of a PDT-treated apoptotic cell (10 μg/ml, upper panel) and necrotic cell fragments (30 μg/ml, lower panel) by macrophages. C127 cells expressing YFP-HSP70 were treated by PDT, then co-incubated with CTxB stained macrophages. The endocytosis of tumor cells or tumor cell remnants (green) by macrophages (red) was clearly shown. Bar = 10 μm. (*b*) Phagocytosis of tumor cells (Vertical-axis) by macrophages (Horizontal-axis) measured by FACS analysis. The population falling within the gated area is shown in the top right quadrant, representing double-stained cells. Cell Tracker Green stained C127 cells treated by PDT with different doses were co-incubated with CTxB stained macrophages for 24 hr. Fluorescent emissions from Green and CTxB were measured by a FACScaner. Cells without PDT treatment incubated with macrophages were used for control. $n = 5$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

by splenocytes was detected 2 days later by ELISA. Stimulated effector cells were tested for cytolytic activity against C127 cells 5 days later using a 4–6 hr cytotoxicity assay (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instruction.

Results

Cell death under different treatments

To confirm the cell death induced by PDT, FACS and Hoechst 33258/PI staining were used. Cells treated by PDT of 10 μg/ml of Photofrin and 5 J/cm^2 of fluence showed significantly apoptotic characteristics, while cells treated with 30 μg/ml of Photofrin and 5 J/cm^2 of fluence showed significantly necrotic characteristics, either analyzed with FACS or fluorescent imaging (Figs. 1*a* and 1*b*). To investigate the kinetics of cell death, time-lapse confocal microscopy was used. Using the relative emission intensity of each fluorochrome as a function of time, we determined the time when individual cells were stained by annexin V or became permeable to PI (Figs. 1*c* and 1*d*). Under the PDT treatment with 10 μg/ml of Photofrin, 5 J/cm^2 of fluence, the majority of cells died within 5 hr, and all showed typical signs of apoptosis. The integrity of the cell plasma membrane was lost following apoptosis, as shown in Figure 1*c*. However, under the PDT treatment of 30 μg/ml of Photofrin, the cells showed a rapid swelling and rupture of the plasma membrane, indicative of necrosis (Fig. 1*d*).

Immunological effects of apoptotic and necrotic tumor cells

PDT-treated cells can be endocytosed by immature macrophages. To determine the endocytosis of apoptotic and necrotic tumor cells, we co-incubated macrophages with PDT-treated cells which express YFP-HSP70. Endocytosis of the tumor cells and the tumor cell remnants by the macrophages was demonstrated by fluorescent images obtained by confocal laser scanning microscopy. Specifically, Figure 2*a* shows the interaction between a macrophage and an apoptotic cell, lasting about 9 hr (upper panel), and the ingestion of fragments of necrotic tumor cells by an immature macrophage, leading to maturation in about 60 min after incubation (lower panel). These results indicate that macrophages are capable of endocytosing PDT-damaged tumor cells and tumor cell fragments.

Macrophages take up more materials from apoptotic cells than from necrotic cells. Flow cytometry analysis was used to further study the endocytosis of damaged cells. PDT-treated C127 cells were co-incubated with macrophages for 24 hr, and analyzed by FACS. When untreated control tumor cells were mixed with macrophages, 2 distinctive populations were detected (Fig. 2*b*), indicating that the macrophages did not appreciably phagocytose the intact tumor cells. In contrast, a high double-stained population was observed (Fig. 2*b*) when the macrophages were incubated with the apoptotic cells, demonstrating phagocytosis of tumor cells by the macrophages. Conversely, only a low level of double-

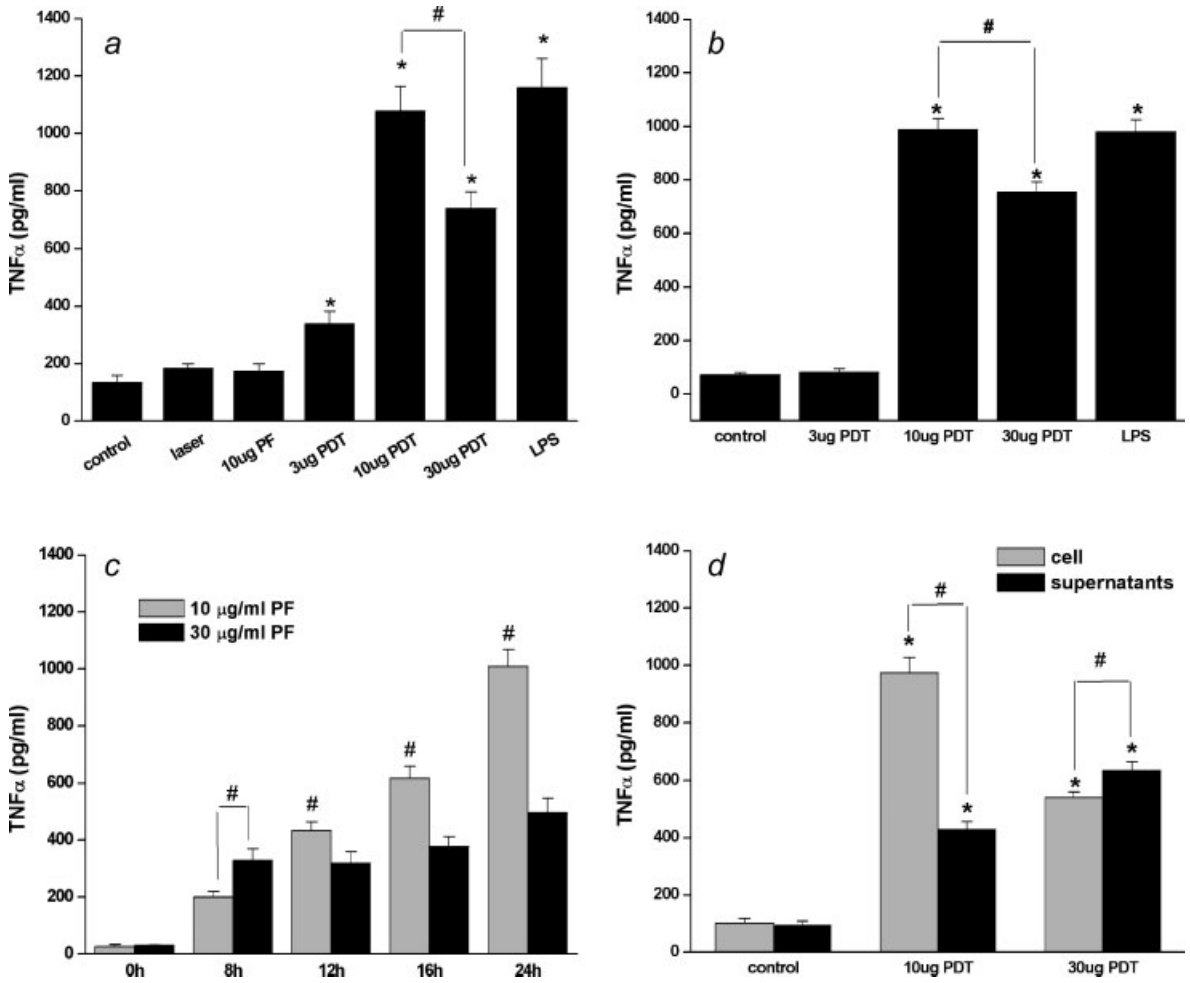

Figure 3 – Cytokine secretion by macrophages co-incubated with tumor cells under different treatments. TNFα secretion by RAW 264.7 macrophage cells (*a*) or mouse peritoneal macrophage cells (*b*) co-incubated with treated C127 cells was detected by ELISA. Macrophages were co-incubated with treated tumor cells for 24 hr (1:1). Cells without treatment incubated with macrophages were used for negative control and LPS stimulated macrophage were used for positive control. After incubation, supernatants were collected for the determination of TNFα. (*c*) TNFα secretion by macrophages was also detected at different times of co-incubation with PDT-treated C127 cells, using ELISA at 0, 8, 12, 16 and 24 hr post-treatment. (*d*) TNFα secretion by macrophages co-incubated with treated C127 cells only and the supernatant detected by ELISA. After the treatment, the supernatants were removed from treated C127 cells, and macrophages were co-incubated with the supernatants and cells, respectively, for 24 hr (1:1). $n = 4$. ($^{*}p < 0.05$ *vs.* control cells; $^{\#}p < 0.05$ *vs.* indicated cells, Student's *t*-test).

stained population was observed (Fig. 2*b*) when the macrophages were incubated with the necrotic cells. These data suggest that the mechanism of cell death can influence the ability of macrophages in detecting and endocytosing tumor cell or tumor cell fragments. Furthermore, the data show that macrophages are more adapted to recognize and clear apoptotic cells than necrotic cells.

Responses of macrophages to different mechanisms of cell death. To determine the responses of macrophages to different types of cell death, we measured TNFα secretions of macrophages, incubated with treated cells for 24 hr and assayed by ELISA. Increased level of TNFα was detected when using cells treated by PDT with a Photofrin dose of 10 μg/ml and 30 μg/ml, or by LPS which used as a positive control, but not when using the cells treated with laser, PF or by PDT with a dose of 3 μg/ml, as shown in Figure 3*a*. In comparison, cells treated with 10 μg/ml of Photofirn were able to stimulate a higher level of secretion of the TNFα than using 30 μg/ml. Similar results were obtained when PDT-treated cells were co-incubated with mouse peritoneal macrophages (Fig. 3*b*).

The co-incubation time affected the macrophage response. As shown in Figure 3*c*, within 8 hr of incubation, the necrotic cells (PDT, 30 μg/ml) stimulated a higher level of the TNFα production, while the apoptotic cells (PDT, 10 μg/ml) induced a higher level of TNFα secretion with a higher rate of increase after incubation of 12 hr.

As shown in Figure 3*d*, after the separation of the apoptotic cells from the medium, the cells alone induced a much higher level of TNFα secretion than the supernatant. On the contrary, the necrotic cells alone induced a lower level of TNFα secretion than its supernatant. These results indicate that the apoptotic cells after PDT treatment are more effective in macrophage activation.

HSP70 regulation of the immune response

TNFα secretion was increased when the macrophages were incubated with PDT-treated C127 cells overexpressing HSP70, either with a Photofrin dose of 10 μg/ml or 30 μg/ml, after 24 hr, as shown in Figure 4*a*. However, the TNFα secretion was at the level of control if the macrophages were incubated with PDT-treated cells when the HSP70 protein was blocked by HSP70 ploYclonal antibody (Fig. 4*a*).

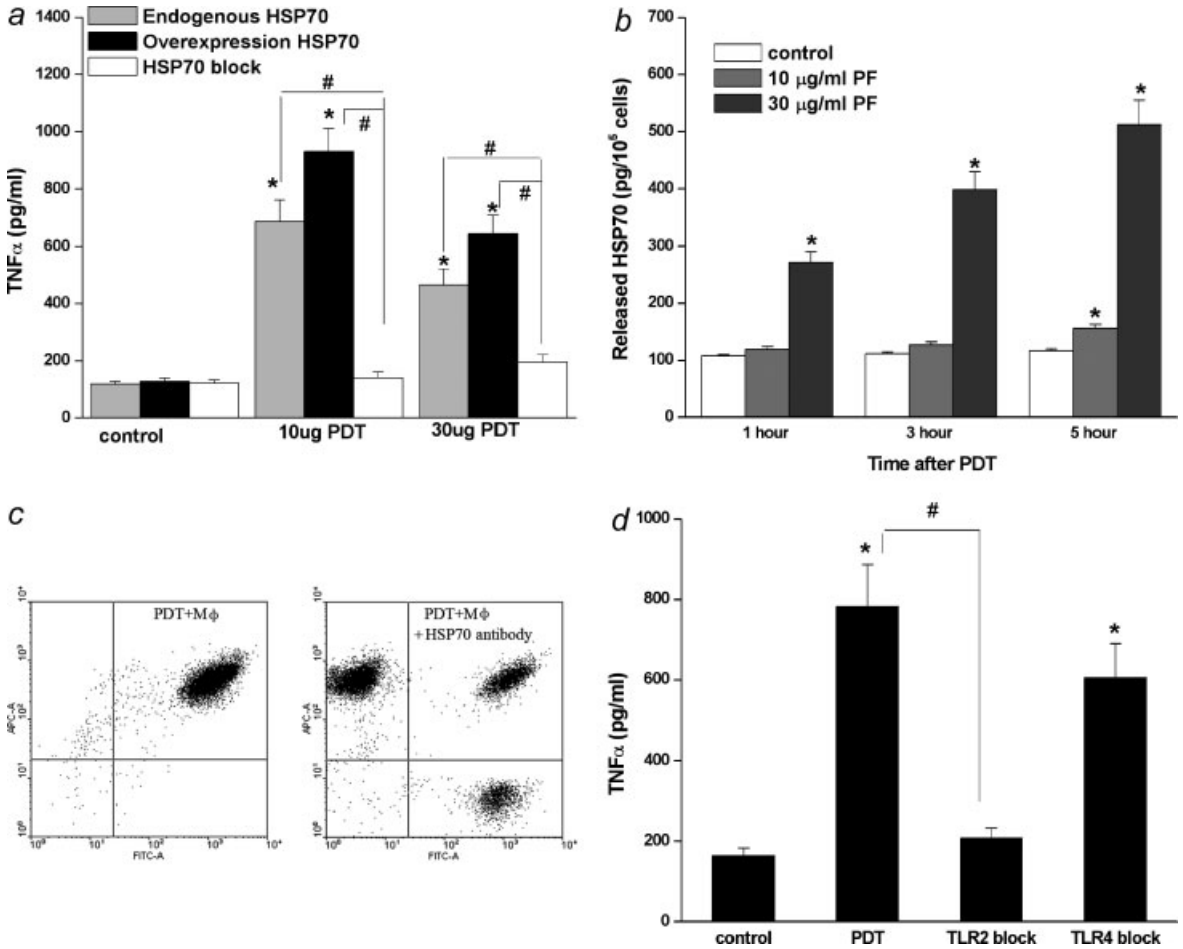

FIGURE 4 – Responses of HSP70 to apoptosis and necrosis induced by PDT. (*a*) TNFα secretion by macrophages co-incubated with C127 cells treated by PDT was detected under different HSP70 regulations (normal expression, gray bar; overexpression, black bar; and with HSP70 antibody, blank bar). Different HSP70 regulations were realized by co-incubating macrophages with control tumor cells, cells expressing YFP-HSP70, and with cells plus HSP70 antibody. Supernatants were collected for the determination of TNFα using ELISA. (*b*) HSP70 release from C127 cells treated by PDT was detected by ELISA. Following the indicated post-treatment times, the cell culture supernatants were collected for the determination of HSP70 release. (*c*) Blocking HSP70 inhibits phagocytosis of apoptotic cell debris. The macrophages co-incubated with apoptotic C127 cells with and without HSP70 antibodies for 24 hr were analyzed by FACS. $n = 5$. (*d*) TNFα secretion by macrophages co-incubated with C127 cells treated by PDT (10 μg/ml), with either TLR-2 or TLR-4 antibodies, was detected. $n = 4$. ($^*p < 0.05$ *vs*. control cells; $^\#p < 0.05$ *vs*. indicated cells, Student's *t*-test).

To further demonstrate the regulation of HSP70 on the macrophage responses to PDT-treated cells, we detected the release of HSP70 due to different treatments (with a Photofrin dose of 10 μg/ml or 30 μg/ml) using HSP70 ELISA. HSP70 was detected in the culture supernatants of cells treated with the necrotic PDT dose (30 μg/ml), but not in the cells treated by apoptotic PDT (10 μg/ml), as shown in Figure 4*b*.

Consistent with the results in Figure 2*b*, co-incubation of the macrophages with tumor cells treated by the apoptotic PDT generated a high proportion of double-stained cells (Fig. 4*c*), indicating phagocytosis of the apoptotic cells. However, when the HSP70 polyclonal antibody was added, the proportion of the double stained macrophages was significantly reduced (Fig. 4*c*). The same phenomenon was not observed in cells treated with the necrotic PDT dose (data not shown).

To investigate how HSP70 affects macrophage response to treated cells, we detected the TNFα secretion of the macrophages co-incubated with cells treated by the apoptotic PDT dose, plus TLR2 or TLR4 antibodies. When obstructing the interactions of HSP70 with TLR2, the TNFα production by the macrophages was significantly decreased (Fig. 4*d*). These results suggest that HSP70 provides a TLR2 dependent signal, to activate macrophages to uptake the damaged cell bodies.

Real-time detection of HSP70 distribution in living cells after PDT treatment

To investigate the impact of apoptotic PDT treatment on HSP70, we monitored the dynamic distribution of HSP70 in real time. After PDT, fluorescence emission from cells expressing YFP-HSP70 showed a significant shift from inside the cell to the cell surface in 30 min (Fig. 5*a*). The control C127 cells expressing YFP-HSP70 showed a stable YFP fluorescence emission inside the cell for more than 4 hr (Fig. 5*a*). Fluorescence intensities of YFP from cells after the PDT treatment showed a continuous decrease with time inside the cytoplasm and a continuous increase on the cell surface, reaching a stable stage after 40 min (Fig. 5*b*).

Furthermore, we found that the rate of HSP70 increase on the surfaces of C127 cells treated by apoptotic PDT depended on the photosensitizer dose (Fig. 5*c*). The higher the PF dose, the faster the translocation of HSP70 to the cell surface.

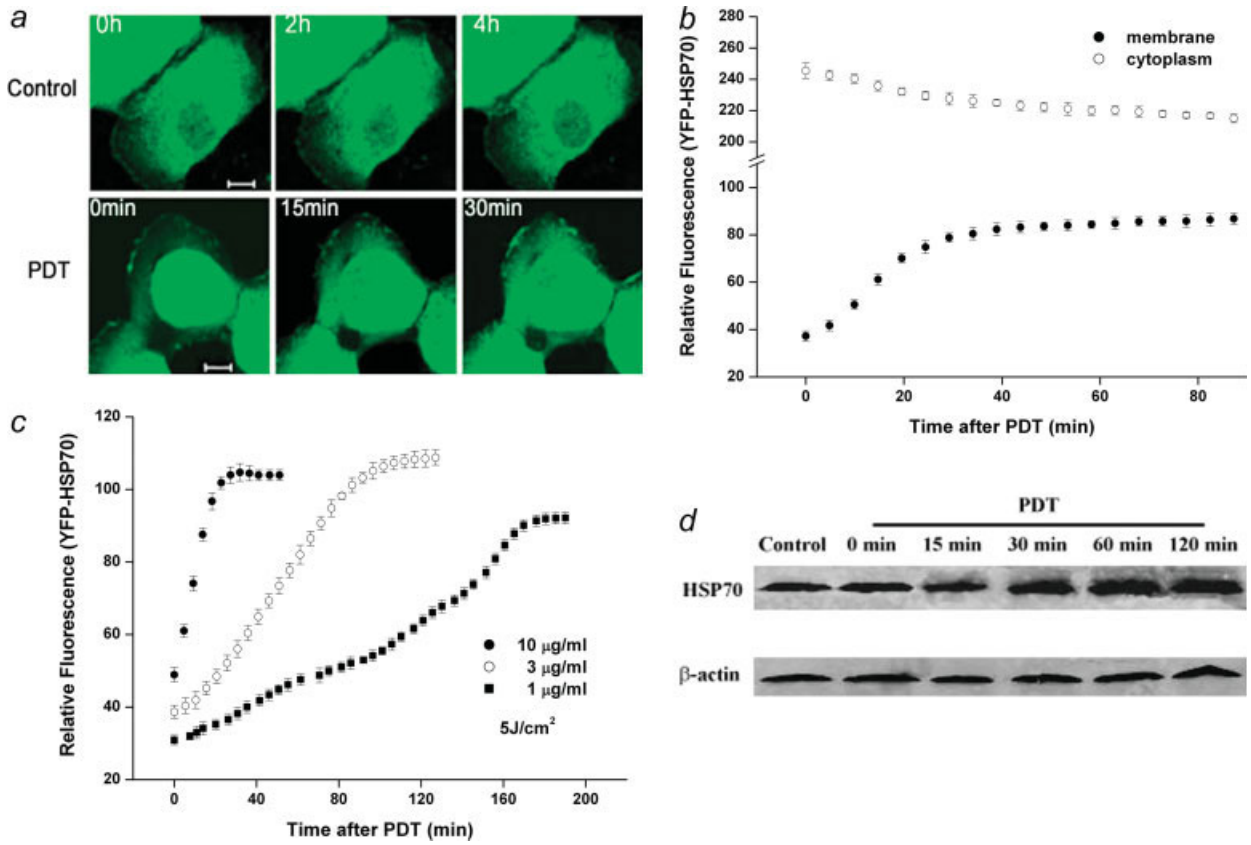

FIGURE 5 – Dynamic changes of HSP70 distribution in a single cell after PDT treatment. (*a*) Fluorescent image series of C127 cells expressing YFP-HSP70 after PDT treatment. Cells were incubated with Photofrin (10 μg/ml) for 20 hr, then treated with 633 nm light (5 J/cm^2), and observed by a confocal microscope. Bar = 10 μm. (*b*) Temporal profiles of YFP emission intensities from C127 cell cytoplasm and membrane. Bars, SD (n = 4). (*c*) Temporal profiles of YFP emission intensities from C127 cell surface after PDT treatment with different Photofrin doses (1, 3, 10 μg/ml, 5 J/cm^2). (*d*) Western blot analysis of cells at different time after PDT treatment was performed to detect the level of HSP70, using β-actin as markers of the proteins in cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The expression of HSP70 in cells was monitored under PDT treatment using western blotting analysis. The results are shown in Figure 5*d*. Compared to control cells (lane 1), total HSP70 levels are elevated following PDT treatment (Fig. 5*a*).

Vaccination of mice with treated tumor cells induces secretion of type 1 cytokines and stimulates the generation of specific CTLs

Five days after immunization with PDT-treated tumor cells, mouse splenocytes were harvested and co-incubated with mitomycin-C-treated C127 cells. Splenocytes primed *in vivo* by vaccination with apoptotic and necrotic (with a Photofrin dose of 10 μg/ml or 30 μg/ml) tumor cells responded with increased secretion of TNFα upon *in vitro* restimulation (Fig. 6*a*). We also found that vaccination with the apoptotic and necrotic cells resulted in the generation of potent CTL activity against C127 cells (Fig. 6*b*). The apoptosis apparently stimulated a stronger immunological response than necrosis (Figs. 6*a* and 6*b*). In contrast, vaccination with cells treated with low dose PDT (3 μg/ml) failed to secrete TNFα and generate CTLs against C127, as shown in Figure 6.

Discussion

Apoptotic or necrotic tumor cells are a rich source of antigens. The immunomodulatory effects by dying tumor cells on the functions of APCs are currently being debated.[31,32] Specifically, immunological responses induced by apoptotic and necrotic tumor cells, regulated by HSP70 during PDT treatment, are not clear. In this study, we investigated the relationships between macrophage responses, HSP70 release and HSP70 translocation, during Photofrin–PDT-induced apoptosis and necrosis. We selected 2 typical dosages of PDT to induce apoptosis and necrosis (Figs. 1*a* and 1*b*) to study different immune responses induced by cell death. Our current findings indicate that macrophages can directly endocytose apoptotic tumor cells and necrotic tumor cell fragments (Fig. 2*a*), and take up more material from PDT-treated apoptotic cells than from necrotic cells (Fig. 2*b*), consistent with results published by Gough *et al.*[17] It was reported that rapid phagocytosis of apoptotic debris ensured that the contents of cells were neatly and safely removed by scavenger cells, without inducing inflammatory responses.[33] In contrast, our results show that the macrophages co-incubated with the cells treated by the apoptotic PDT dose (10 μg/ml), as well as with the cells treated by the necrotic PDT dose (30 μg/ml), were activated to produce TNFα, as shown in Figure 3*a*. Furthermore, the level of macrophage activation by cells treated with apoptotic PDT dose was higher than that by necrotic cells (Fig. 3*a*). Similar results were obtained when PDT-treated cells were co-incubated with mouse peritoneal macrophages (Fig. 3*b*). These results indicate that PDT-induced apoptotic cells can trigger the maturation of macrophage. However, it is unknown how PDT-induced apoptotic cells could mature the macrophage.

Macrophages are capable of discriminating apoptotic cells and necrotic cells and respond accordingly. It is reported that the difference between immunological outcomes induced by the apoptotic and necrotic cells is not due to the differential uptake of materials by the macrophages, but rather due to whether and how the

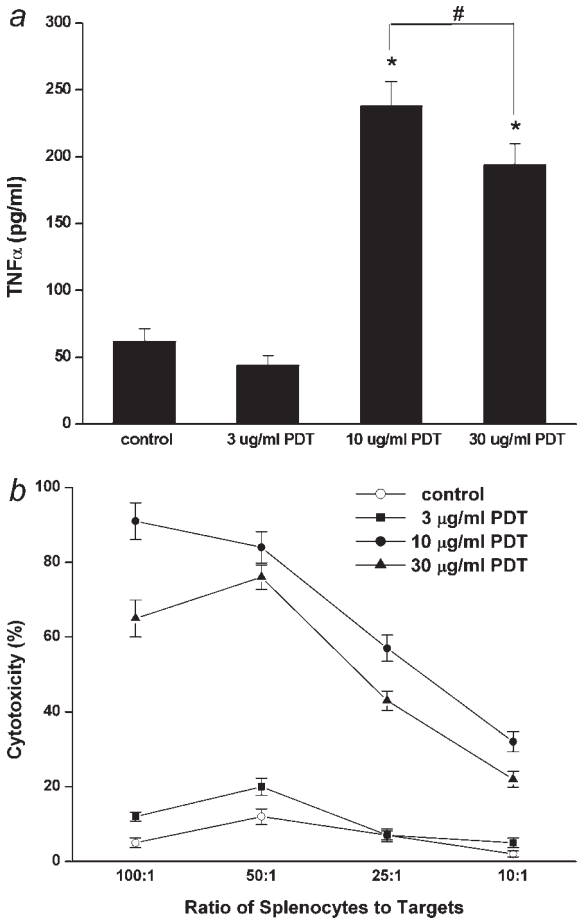

FIGURE 6 – Immunization of mice with PDT-treated cells induces tumor-specific CTLs. BALB/c mice were immunized with cells treated by PDT of different doses (100,000 cells/ animal) and mouse spleen cells were harvested 5 days later. (*a*) TNFα secretion of spleen cells harvested from immunized mice. Harvested spleen cells were restimulated with mitomycin C-treated C127 cells for 2 days and were tested for TNFα secretion by ELISA. The increased TNFα secretion induced by PDT-treated cells is clearly demonstrated. $n = 4$. ($^*p < 0.05$, Student's *t*-test). (*b*) Tumor cytotoxicity of spleen cells from immunized mice. Harvested spleen cells were restimulated with mitomycin C-treated C127 cells for 5 days and were tested for cytolytic activity against C127 cells using a 4–6 hr cytotoxicity assay ($n = 4$).

macrophages are matured and activated following the phagocytosis of dying cells.[33] Necrotic cells were believed to release highly inflammatory noxious intracellular contents, pinocytosed by macrophages, inciting inflammatory responses.[34,35] In comparison, after engulfing apoptotic cells macrophages were commonly believed to have anti-inflammatory and immunosuppressive responses if the engulfed apoptotic cells maintained intact membranes.[2,3] If the apoptotic cells become permeabilized (often referred to as "secondary necrosis"), they can switch the macrophages' responses from anti-inflammatory to pro-inflammatory.[18] Our study showed that within 8 hr of incubation with the macrophages, the necrotic cells stimulated a higher level of TNFα production than the apoptotic cells, while after the incubation of more than 12 hr, the apoptotic cells induced more TNFα secretion with a higher rate of increase, as shown in Figure 3*c*. We also observed that the apoptotic cells alone induced a much higher level of TNFα secretion than its supernatant, while the necrotic cells alone induced a lower level of TNFα secretion than its supernatant (Fig. 3*d*). It was also shown that the apoptotic bodies eventually became permeabilized (Fig. 1*c*), indicating that such secondary necrosis may release danger signals to stimulate macrophages for TNFα secretion. It is also reported that apoptosis induced by heat stress stimulates dendritic cells and induces specific cytotoxic T cells.[33] Photooxidative lesions produced in PDT-treated tumors can also be recognized by the host as "altered self," hence prompting the inflammatory and immune responses.[21]

Furthermore, we studied whether HSP70 could affect the immune response triggered by PDT-induced apoptotic cells, since HSP70 translocated to the cell surface during apoptosis induced by PDT.[21,30] The macrophages incubated with the PDT-treated tumor cells expressing YFP-HSP70 produced a significantly high level of TNFα, while the macrophages incubated with the PDT-treated tumor cells produced a significantly low level of TNFα (Fig. 4*a*), if HSP70 was blocked. These findings strongly support the pivotal role of HSP70 on the immune response induced by PDT. We also showed that HSP70 released from the necrosis tumor cells, engulfed by the macrophages, could regulate the macrophage activation and the TNFα production (Figs. 3*d* and 4*b*). However, we did not observe the HSP70 release from the apoptotic cells (Fig. 4*b*).

When HSP70 was blocked by HSP70 antibody during co-incubation of the macrophages with the PDT-treated apoptotic cells, the interaction between tumor cells and macrophages was inhibited, decreasing the antigen engulfment by macrophages (Fig. 4*c*). It suggests that HSP70, although not released from the cell, still provides a specific signal to activate macrophages to uptake the apoptotic bodies. In our previous studies, we observed the dynamic translocation of cytoplasmic HSP70 onto the surface of a single cell during the PDT-induced apoptosis.[30] Fluorescent imaging (Fig. 5*a*) and spectrofluorometric analysis (Fig. 5*b*) showed an instantaneous increase in the YFP emission from the cell surface and a corresponding emission decrease from cytoplasm, reflective of the translocation of HSP70 onto the cell surface. The extent of the initial surface HSP70 expression depends on the PDT dose (Fig. 5*c*). Figure 5*d* clearly shows that the expression of HSP70 in cell started increase 30 min after PDT treatment, which could protect cells. However, the surface translocation of HSP70 occurred earlier, indicating that the translocation of HSP70 was the initial stress to protect the cell membrane under PDT treatment.[30]

It is reported that the COOH-terminal domain of HSP70 is exposed when it is expressed on the cell surface, and the COOH-terminal domain is involved in the interaction of HSP70 with its chaperoned endogenous peptides,[23,36,37] hence, damaged cell proteins are more likely to be attached to the substrate-binding domain of HSP70 that may serve as a source of tumor antigens.[38] Besides their chaperone activity, HSP-peptide complexes are internalized into antigen-presenting cells by a receptor-mediated endocytosis, then channel them into the MHC class I presentation pathway of antigen-presenting cells.[39,40] A-macroglobulin receptor (CD91) is a major receptor responsible for endocytosis of released HSPs.[41] However, a number of other molecules on the cell surface were also identified as receptors for HSPs, including TLR2 and TLR4 with their co-receptor CD14, scavenger receptor (CD36) and co-stimulatory molecule CD40.[39] Engagement of TLR2 and TLR4 driven by HSP70 is now recognized as a major route of activation of dendritic cells and other antigen-presenting cells, as well as the means of upregulation of numerous genes relevant to immune responses.[21,41,42] Our results show that the macrophages incubated with the PDT-treated tumor cells produced a significantly low level of TNFα, when TLR2 was blocked (Fig. 4*d*), indicating that the HSP70-stimulated macrophage response is TLR2 dependent. Furthermore, when the HSP70 was blocked, the level of TNFα induced by apoptotic PDT treatment was at the same level of the control (Fig. 4*a*), while when the TLR2 was blocked, the level of TNFα induced by apoptotic PDT treatment was only slightly higher than the level of the control (Fig. 4*d*). These results demonstrated that, although TLR2 was not the only receptor for HSP70 it was indeed the dominating receptor. Our results are consistent with the strong HSP70-TLR2 interaction reported previously.[21,41]

These results suggest that HSP70 is quickly expressed on the surface under the PDT-induced apoptosis, presenting potent endogenous danger signals to the macrophages through the TLR2 receptor and then inducing inflammation (Figs. 3*a* and 5*b*). However, cells treated by low PDT doses also expressed HSP70 on cell surface but without inducing TNFα production of the macrophages (Figs. 3*a* and 5*c*). HSPs have been reported to be used by cells to reinforce the membranes in the case of their destabilization.[43] Other reports have suggested that HSP70 is anchored at the cell surface as a part of a larger molecular complex and may serve as certain surface receptors,[44] which chaperoned endogenous peptides that act as stimulant for macrophage activation. Our experimental results of non-lethal PDT doses support the reinforcement role of HSP70,[30] while our results of lethal PDT doses support the immunological activation role of HSP70. These results further suggest that HSP70 expression on cell surface alone can enhance the macrophage's ability to uptake the apoptotic bodies (Fig. 4*c*), and combined with the danger signals, such as in the case of the PDT-induced apoptosis and necrosis, HSP70 clearly became a key molecule to activate the macrophages, as shown Fig. 4*a*.

The production of type 1 cytokines and the generation of tumor-specific cytotoxic T cells characterize *in vivo* immune responses.[33] We showed that splenocytes from mice immunized with PDT-treated cells (Photofrin, 10 or 30 μg/ml; fluence, 5 J/cm^2) secreted significantly higher level of TNFα than spleen cells from mice immunized with cells treated with low PDT dose (3 μg/ml of Photofrin) (Fig. 6*a*). We also presented the *in vivo* evidence that PDT-induced apoptosis can prime CTLs (Fig. 6*b*). In our preliminary animal studies, we demonstrated that apoptotic cells induced higher immune responses than necrosis cells *in vivo* (Fig. 6), consistent with our *in vitro* results (Fig. 3).

In summary, we provided *in vitro* (through macrophages) and *in vivo* (through splenocytes) evidence of immune responses induced by PDT treatment and the regulatory role of HSP70. Our results show that PDT-induced cell damage can provide HSP70, either presented on the surface of the apoptotic cells or released by the necrotic cell fragments, not only to stimulate macrophage phagocytosis, but also to alter macrophage maturation combined with the "danger signal." Among these two "danger signal" producers, apoptosis can induce a higher level immune response because HSP70 presented on the cell surface can increase the material uptake by the macrophages to represent more potent endogenous danger signals and enhance the macrophage activation. These findings could provide a better understanding of PDT-induced immune responses, leading to an improved PDT treatment regimen.

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