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Abstract: Heat shock proteins (Hsps) hold a dual role depending on their location. Inside cells, they fulfill essential survival functions as molecular chaperones forming complexes with intracellular polypeptides (self or foreign) to help in protein folding, the resolution of protein aggregates and intracellular protein transport. Released from the cell, they act as messengers communicating the cells' interior protein composition to the immune system for initiation of immune responses against intracellular proteins. Here we describe the mechanisms by which Hsp70, the heat-inducible Hsp70 family member, crosstalks with the immune system. Further, we discuss that clinical hyperthermia could be a way to initiate the immunologic activity of Hsp70 by upregulating its expression and facilitating release through local necrosis.

Dear Mrs Gebauer,

Thank you for giving us the opportunity of submitting our revised version of the manuscript "Hsp70, a messenger from hyperthermia for the immune system" for publication in the European Journal of Cell Biology.
We have made changes recommended by the reviewer on the printed version of the manuscript, which included the suggestion of additional references as well as the more specified description of figure 4A. Linguistic corrections have also been included as proposed by the reviewer.

We belief our review now fulfils your required standards and hope it will be accepted for publication.

Sincerely,
Elfriede Noessner, on behalf of all authors

***Response to Reviewer Comments**

Response to reviewer

Thank you for your helpful comments.
We have included the linguistic corrections as you suggested. Furthermore we added additional references (Milani et al. 2009) and specified the description of figure 4A.

Thank you for taking the time of reviewing our manuscript.

Sincerely,
Elfriede Noessner, on behalf of all authors

Hsp70, a messenger from hyperthermia for the immune system

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Abstract

Heat shock proteins (Hsps) hold a dual role depending on their location. Inside cells, they fulfill essential survival functions as molecular chaperones forming complexes with intracellular polypeptides (self or foreign) to help in protein folding, the resolution of protein aggregates and intracellular protein transport. Released from the cell, they act as messengers communicating the cells' interior protein composition to the immune system for initiation of immune responses against intracellular proteins. Here we describe the mechanisms by which Hsp70, the heat-inducible Hsp70 family member, crosstalks with the immune system. Further, we discuss that clinical hyperthermia could be a way to initiate the immunologic activity of Hsp70 by upregulating its expression and facilitating release through local necrosis.

Keywords: Heat shock protein 70, hyperthermia, NK cells, Hsp70-specific CD4 T cells, IL-10, crosspresentation

Hyperthermia, heat shock proteins and immune activation

The interest to study the human heat-inducible heat shock protein (Hsp)70 and its crosstalk with cells of the immune system originated from the desire to provide a mechanistic understanding for the observation that clinical hyperthermia in the combined application with chemotherapy or radiation not only helps to control local tumor recurrence but also reduces the occurrence of distant metastasis in patients with melanoma, recurrent breast carcinoma and cervival cancer and high-risk soft tissue sarcoma (Issels et al. 2010; and *refs* therein). Classically, the application of hyperthermia in the treatment of cancer is rationalized by the knowledge that heat increases the efficacy of chemotherapy and radiation (Wust et al., 2002; Issels, 2008) and, above a certain threshold, can also directly kill tumor cells. These direct effects, however, do not satisfactorily explain the observed systemic effects controlling occurrence of distant metastasis. Early observations in mouse models suggested a role of cell-mediated immunity in the hyperthermia-mediated tumor control, most likely through a macrophage-T cell interaction (Alfieri et al., 1981). Considering Hsps as mediators in this response is based on work by Srivastava et al. (Srivastava, 2002; Udono and Srivastava, 1993), that had provided evidence in mouse tumor models, that vaccination with stress proteins, gp96 and Hsp70, isolated from mouse cancer tissue induced cancer-specific immunity and antigen-specific cytotoxic T lymphocyte activation by a mechanism that involved antigen-presenting cells (APCs).

Hsps are a large group of conserved and ubiquitous intracellular proteins. In the normal cellular environment, they perform essential survival functions as molecular chaperones with high intrinsic binding to hydrophobic polypeptides thereby mediating protein folding, resolution of aggregates and the translocation of proteins between different intracellular compartments (Mayer and Bukau, 2005; Wegele et al., 2004). To interact with the immune system, Hsps have to change location from their classical intracellular to the extracellular environment. Details of this translocation remain to be fully elucidated, since classical

secretion signals are absent. Documented mechanisms include the release by cell necrosis or in response to cellular stress (*refs* in Bendz et al., 2007). Elevated temperature, as reached during hyperthermia, is one type of cell stress. It causes proteins to become structurally labile and unfold, leading to intracellular accumulation of aggregates and thereby initiating the "stress response" with cells producing large amounts of molecular chaperones (Ellis, 1987; Fuller et al., 1994; Walters and Buchner, 2002). Heat can also cause necrosis, allowing the release of Hsps to the extracellular environment. Among the different Hsps, Hsp70 is the one which is most strongly induced by heat. Thus, it was the chaperone of interest to study in the context of hyperthermia.

The presumed effects of regional hyperthermia on the tumor and the immune response are summarized in **Figure 1**. Research over the last 12 years, made possible through funding provided by the German Research Foundation (SFB455), provided mechanistic insight into these postulated events.

Hsp70 as activator of and recognition structure for NK cells

Work by Multhoff et al. (2007) has shown that after a non-lethal heat-shock Hsp70 was found to be expressed on the surface of malignant cells, facilitating their recognition and lysis by NK cells. In a mouse model, Hsp70-activated NK cells were shown to control Hsp70-positive primary or metastatic cancer (Stangl et al., 2006) and, in a clinical phase 2 study, to generate immune responses in patients (Krause et al., 2004; Milani et al., 2009). The mechanistic details behind these observed effects are not fully resolved. We have shown that Hsp70 does not induce calcium signals in NK cells (**Figure 2**), thus NK-activating receptors, such as NKp30, NKp46, NKp44 are not likely involved. CD94, a non-calcium signaling NK cell receptor, has been postulated as a putative Hsp70 receptor leading to NK cell activation (Multhoff, 2007). Hsp70 expressed on tumor-cell surfaces has been described as a recognition structure for NK cells inducing them to release granzyme B leading to tumor-cell apoptosis.

Regarding the mechanism of granzyme B entry, it was speculated that membrane-localized Hsp70 could generate cationic channels allowing access of granzyme B into the cell (Multhoff, 2007).

Hsp70-facilitated antigen crosspresentation to CD8 T cells via dendritic cells relies on the Hsp's proficiency as molecular chaperone

Srivastava et al. (Srivastava, 2002; Udono and Srivastava, 1993, 1994) were the first to demonstrate that immunization with gp96, a glucose-regulated stress protein, or Hsp70 isolated from mouse tumors protected mice from a subsequent challenge with tumor cells. Similar vaccination effects were shown for a number of tumor-derived chaperone proteins (Hsp90, Hsp110, calreticulin) in various animal and human models (Castelli et al., 2004; Milani et al., 2002; Nair et al., 1999; Parmiani et al., 2004; Srivastava et al., 2002; Udono and Srivastava, 1993, 1994). Protection was specific against the tumor from which the Hsp was isolated suggesting that the immune response was induced against tumor-expressed epitopes carried by Hsp not to the Hsp protein itself (Binder et al., 2005; Breloer et al. 1998; Li et al. 2002). We used an in vitro melanoma model to show that Hsp70, isolated from tumor cells, reflected the tumor's antigenic profile. Upon incubation of the melanoma-derived Hsp70 with monocyte-derived immature dendritic cells (DCs), DCs acquired the antigenic profile of the tumor and induced antigen-specific T cell activation via MHC class I molecules (a mechanism called crosspresentation) (Noessner et al., 2002). Together with another report by Castelli et al. (2001), this showed, for the first time in a human system, that Hsp70 isolated from tumor cells carried tumor-expressed antigens and was able to transfer them to DCs for T cell recognition.

The mechanism of Hsp70-mediated antigen transfer to the MHC-class I presentation pathway of DCs was further elucidated using recombinant Hsp70 and synthetic antigenic polypeptides (Melan-A/MART-1, tyrosinase). It was shown that recombinant Hsp70 significantly enhanced

the presentation of antigenic epitopes by DCs, if stable complexes were formed between Hsp70 and the polypeptide (Bendz et al., 2007). Stable complex formation was strongly dependent on the presence of an Hsp70 binding motif within the polypeptide sequence (Pandya et al., 2009). Hsp70:peptide complexes accumulated more efficiently in vesicular DC compartments than peptide alone **(Figure 3)**.

An Hsp-specific cell surface receptor has not been identified, yet. Rather, Hsps appear to interact with miscellaneous cell surface receptors that all have very promiscuous binding properties (Calderwood et al., 2007). We have observed that the uptake of Hsp70 by DCs and B cells was mediated by endocytotic mechanisms without evidence for involvement of any cell surface receptor. Using single-molecule tracing, there was evidence of preferred membrane regions for rhuHsp70 entry into cells. Whether these regions relate to lipid rafts, which have been described as entry ports for Hsp70, remains to be elucidated (Wang et al., 2006).

The controversy regarding Hsp70 as an activator of innate immune cells

The exquisite ability of extracellular Hsps, in particular Hsp70, to activate antigen-specific immune responses is thought to be mediated by the dual property of Hsps to transfer the chaperoned protein-cargo to APCs for crosspresentation (Binder et al., 2001, 2005; Castelli et al., 2001; Noessner et al., 2002; Singh-Jasuja et al., 2000a) and to concomitantly induce APC maturation and cytokine secretion (Asea et al., 2000; Breloer et al., 2001; Kuppner et al., 2001; Singh-Jasuja et al., 2000b), thereby creating the immunogenic environment required for effective induction of adaptive CD8 T cell responses. Indeed, we and others, initially reported evidence that Hsp70 induces DC maturation (Asea et al., 2000; Kuppner et al., 2001; Wallin et al., 2002). These effects involved the activation of toll-like receptors (TLRs) and their downstream signaling pathways (Asea et al., 2000; Vabulas et al., 2002). Suspicion about the contribution of Hsp to this process arose when non-proteinaceous products, such as LPS,

lectins and flagellin, were found to copurify with Hsp70 and other Hsps (Bausinger et al., 2002; Tsan and Gao, 2004; Wallin et al., 2002; Ye and Gan, 2007). These substances are ligands for TLRs, such as TLR2, TLR4 or TLR5, which efficiently stimulate DCs to upregulate phenotypic markers and to secrete chemokines and cytokines. Subsequent experiments with endotoxin-depleted Hsps performed by us and others failed to detect signatures of Hsp70-induced DC maturation, leading to the conclusion that contaminating endotoxins and not the Hsp protein itself had caused the stimulatory effects on DCs (discussed in Bendz et al., 2007, 2008).

Subsequently, we identified ADP/ATP nucleotides as an additional class of contamination. Nucleotides have been shown to cause calcium signals in DCs (see *refs* in Bendz et al., 2008). Calcium signals associated with Hsp70-stimulated immune responses have been documented (Floto et al., 2006; Whittall et al., 2006). We found that calcium signals induced in DCs using commercial preparations of human Hsp70 and mycobacterial Hsp70 were due to contaminants. No calcium signals were observed using our in-house prepared highly purified and nucleotide-depleted Hsp70 which still fulfilled its function to enhance antigen presentation (Bendz et al., 2008).

As chaperones, Hsps have a high intrinsic binding capacity for hydrophobic protein sequences, nucleotides and endotoxins (Habich et al., 2005; Mayer and Bukau, 2005; Triantafilou and Triantafilou, 2002). Thus, Hsp preparations are prone to "contamination" with proteins and non-proteinous substances. Obtaining Hsp preparations without immunoreactive by-products is a challenging, maybe impossible, endeavor. While this hampers the conclusions we can draw from in vitro systems regarding the Hsps' capacity to initiate innate immune signals, it does not exclude that Hsps may deliver important signals to innate immune cells in vivo. After all, the presence of these nominally intracellular proteins in the extracellular space is "abnormal", a sign of cell death, or tissue damage, and of a possible threat to the organism. Thus, released protein, such as Hsps, ringing the alarm bell to mobilize

a rescue system does not seem implausible. Hsps, with their inherent property as chaperones, will arrive in the extracellular space with an array of bound peptides (self and foreign) that were generated within the cells before the Hsp was released. Thereby, Hsps would not only announce the presence of danger, they would also inform the immune system about the identity of the enemy.

Hsp70:peptide complexes allow de novo priming of antigen-specific CD8 T cells concomitant with the activation of Hsp70-specific CD4 T cells

As described above, we found that highly purified endotoxin- and nucleotide-depleted human Hsp70 significantly enhanced crosspresentation of exogenous antigens by means of complex formation and augmenting antigen uptake (**Figure 3**). To address the question whether the enhanced delivery and crosspresentation of antigen to APCs would translate into stronger antigen-specific T cell responses, priming experiments were performed. Melanoma-associated antigen Melan-A/MART-1 was used as the antigenic epitope. After two rounds of incubation of peripheral blood lymphocytes with Hsp70:MART-peptide complex-loaded DCs or peptide-loaded DCs without Hsp70, the frequency of MART-specific CD8 T cells was determined. While the total percentage of CD8 T cells was similar in both priming conditions, more MART-reactive CD8 T cells were found within the CD8 population (19.5%), if the priming was performed using DCs loaded with Hsp70:peptide complexes, compared to 14% in priming cultures with DCs loaded with MART-peptide alone (**Figure 4A**).

Concomitant with the activation of CD8 T cells against the chaperoned peptide, induction of an Hsp70-reactive T cell response was observed. The recognition was MHC-class II-restricted suggesting an involvement of a CD4 T cell population (**Figure 4B**).

T cells reactive against chaperones are frequently detected in patients with inflammatory disease (van Eden et al., 2005), such as rheumatoid arthritis and diabetes, where they have

been associated with inflammation suppressive effects, by a mechanism involving the production of IL-10 (Prakken et al., 2001).

In our priming cultures, pro-inflammatory Th1 (IFN-γ, TNF-α) as well as anti-inflammatory Th2 (IL-4, IL-5) cytokines and IL-10 were detected in response to MHC-class II-presented Hsp70 (**Figure 4B**). The frequency of each T cell population as well as their contribution to the antitumor response has to be determined in future experiments.

Conclusions and future perspectives

The years of research strengthened the evidence that the immunologic processes proposed to be activated by hyperthermia (see Figure 1) can be performed by Hsp70 in vitro. Whether these principles are involved in the clinical benefit of hyperthermia remains to be established. Monitoring these events in vivo will be a challenging endeavor. Confirmed events are upregulation of Hsp70 expression and local necrosis in tumor tissue after hyperthermia. In a next step, one might consider analyzing whether activated NK cells are seen in proximity to Hsp70-positive tumor cells in hyperthermia-treated tumors. Additionally, one might consider monitoring the emergence of Hsp70-reactive CD4 T cells after hyperthermia treatment which would suggest that Hsp70 was released from tumor cells and presented by DCs (or other APCs) and that the Hsp70-loaded DCs gained access to the T cell repertoire. Regarding the clinical benefit achieved with hyperthermia, these will be exciting topics to be resolved in the future.

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Figure legends

Fig. 1: Working hypothesis of hyperthermia. Elevated temperatures reached through clinical hyperthermia can upregulate Hsp70 expression in tumor cells. Hsp70 can be expressed on the cell surface of some tumor cells acting as a recognition structure for NK cells. Within this temperature range, local necrosis occurs resulting in the release of Hsps and Hsp:peptide complexes. Uptake by DCs and subsequent processing and presentation of the Hsp-bound peptides allows antigen-specific priming of circulating T cells.

Fig. 2: Hsp70 does not induce calcium signals in NK cells. NK cells were stained with Indo-1 and the fluorescence was measured at 510 nm (Fl7) and 420 nm (Fl8) using the Moflow cytometer. Fluorescence was measured for 1 minute to determine the baseline calcium level in NK cells. Then, 0.75 µg/ml ionomycin or 200 µg/ml Hsp70 was added (arrow). An increase in intracellular calcium, as seen after ionomycin addition, leads to a shift in the fluorescence ratio Fl8/Fl7.

Fig. 3: DCs internalize more peptide if peptide is provided as Hsp70:peptide complex. (A) Confocal image of a DC (red: surface HLA-class I proteins) with internalized Hsp70:peptide complexes (green). Cell nuclei are depicted in blue. (B) Flow cytometry of DCs after incubation with fluorescently (FL) labeled peptide or FL-peptide complexed to Hsp70. Histograms show higher fluorescence intensity when DCs were incubated with the Hsp70:peptide complex than with peptide alone.

Fig. 4: Hsp70:MART-complex loaded DCs prime MART-specific CD8 T cells and Hsp70-specific CD4 T cells. (A) Peripheral blood T cells were incubated with autologous DCs loaded either with MART-peptide alone or Hsp70:MART-peptide complex. After two

rounds of stimulation, the induced T cell populations were analyzed for the frequency of MART-specific CD8 T cells. The frequency of MART-reactive T cells within the CD8 T cell population was analyzed using the degranulation assay (Betts et al., 2003). Thereby, the primed T cell populations were cocultured with a melanoma cell line, MEL624.38 which expresses the MART-antigen, for 8 h in the presence of FITC-labeled anti-CD107a/b antibodies and analyzed by flow cytometry. Depicted are the histograms of CD107-FITC, gated on the $CD3^{+}CD8^{+}$ T cells, of the T cell priming with Hsp70:MART-complex-loaded DCs (left) and the T cell priming culture with MART-loaded DCs in the absence of Hsp70 (right). Numbers indicate the percentage of $CD107^{+}$ CD8 T cells (which are the MART-specific T cells) in respective priming cultures. Respective unstimulated T cell cultures were used to define the region of the $CD107^{+}$ cells. (**B**) T cell cultures, primed with either DCs alone (grey bars) or DCs loaded with Hsp70 (black bars), were incubated with either DCs alone or DCs presenting Hsp70 (Hsp70-DCs), in the presence or absence of MHC-class II-blocking antibody. After 24 h, supernatants were harvested and the cytokine content measured using the Th1/Th2-multiplex array (Bioplex, Bio-Rad). Bars are the mean of duplicate values.

Figure 1

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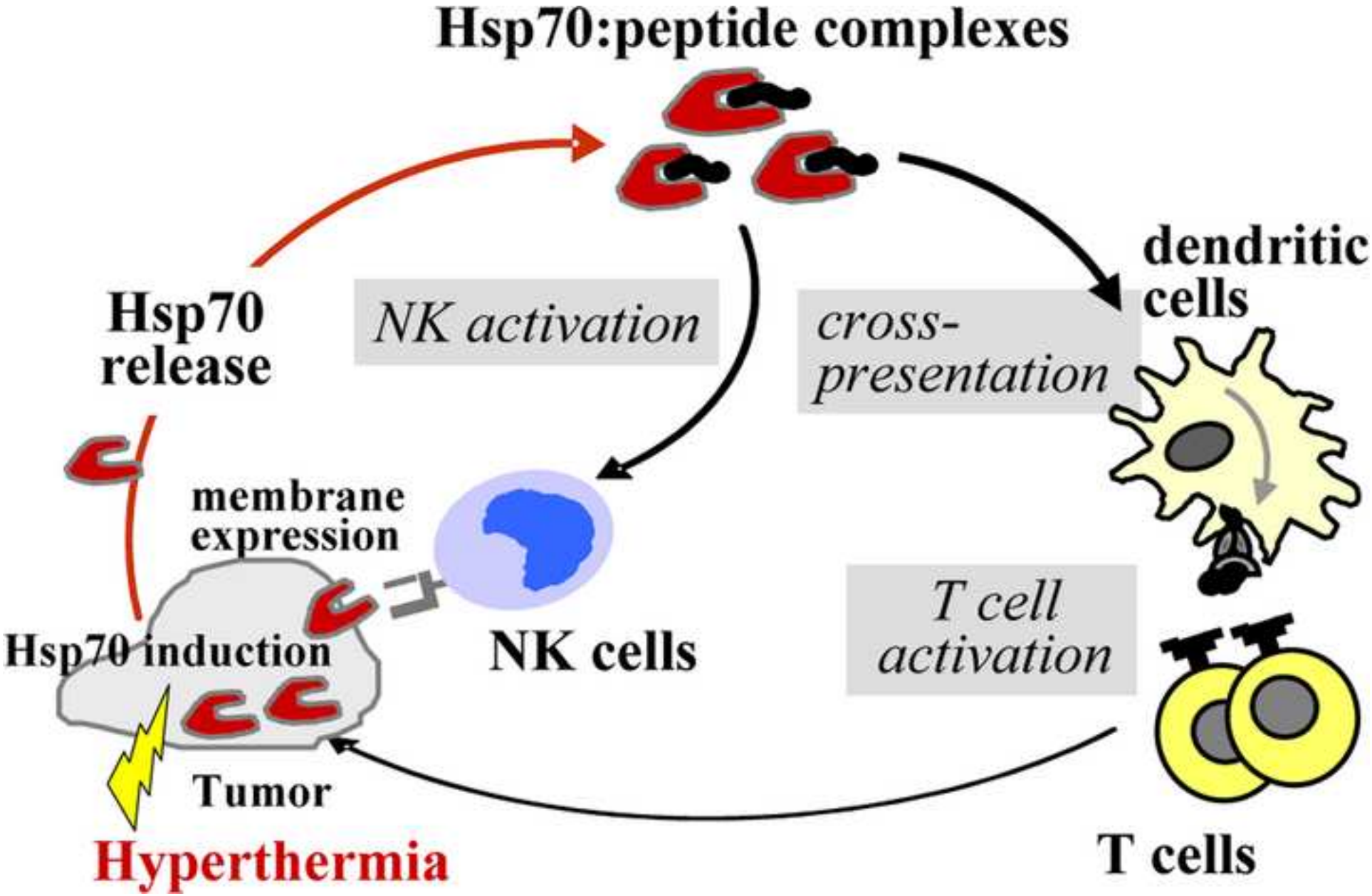

Figure 2

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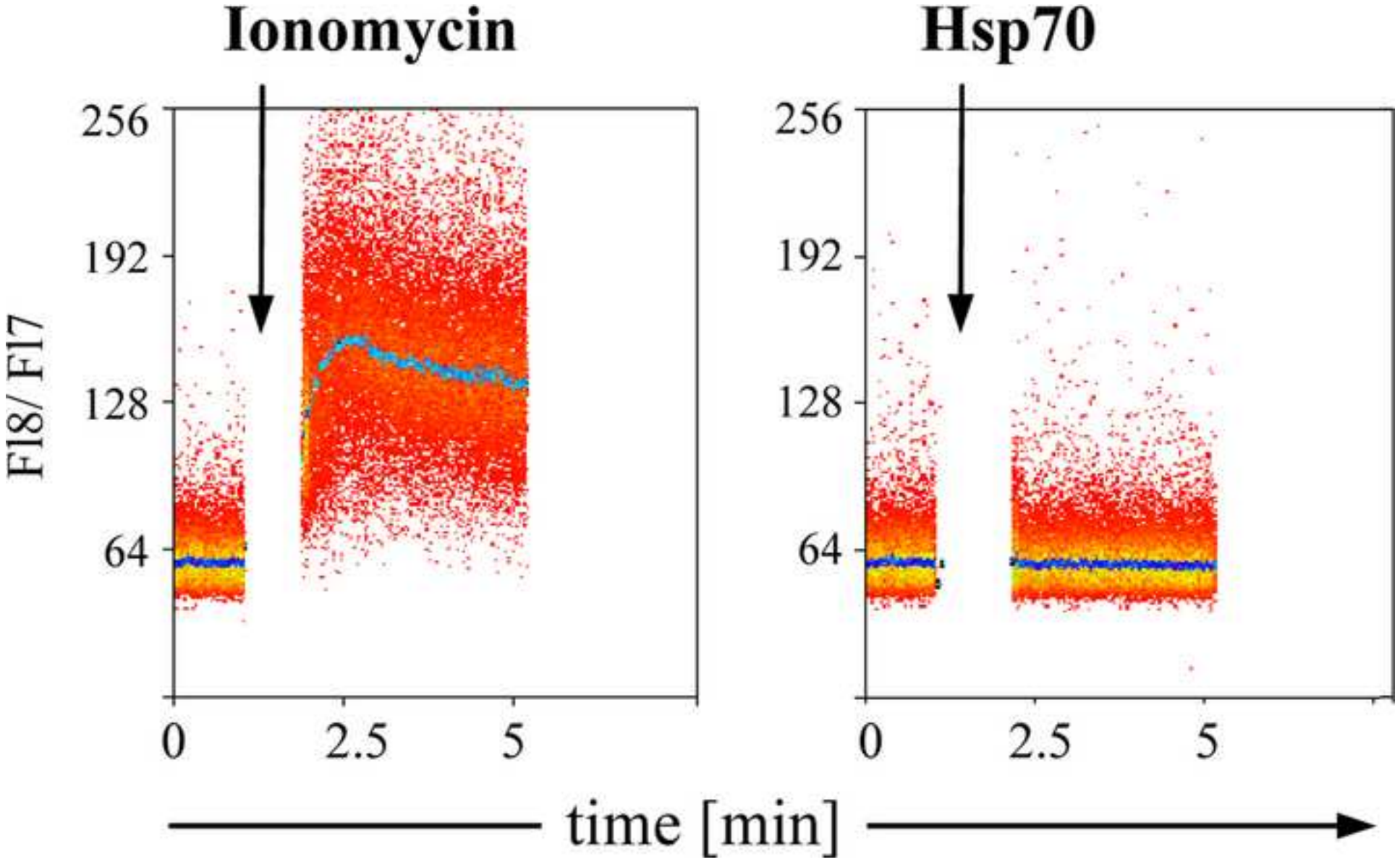

Figure 3

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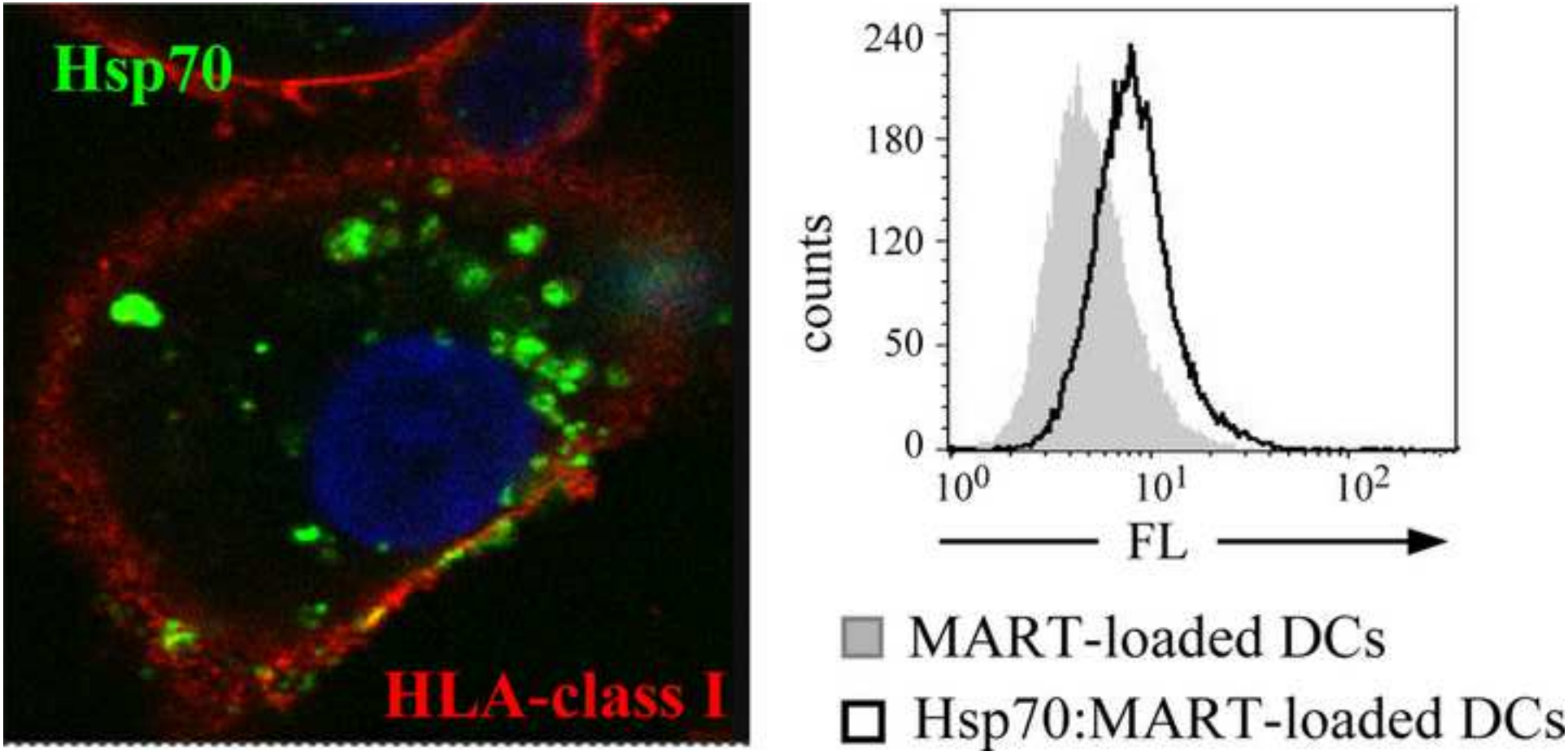

Figure 4

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