The role of heat shock protein (hsp70) in dendritic cell maturation: Hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors

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Members of the heat shock protein (hsp70) family are either constitutively expressed (hsc70) or can be induced by hyperthermic stress (hsp70). Recombinant hsp70 (rhsp70) stimulates cytokine production from monocytes and enhances NK cell proliferation and cytotoxicity. Here we demonstrate that rhsp70 binds to immature dendritic cells (DC) derived from monocyte precursors and induces their maturation as evidenced by an increase in CD40, CD86 and CD83 expression. Immature DC stimulated to mature with rhsp70 show an enhanced ability to present tyrosinase peptide to specific CTL. Mature DC did not bind rhsp70, suggesting a down-regulation in the expression of its receptor. When rhsp70 was added to monocyte precursors at the same time as GM-CSF and IL-4 it reduced the differentiation of monocytes into DC as shown by a decrease in the level of CD40, CD83, CD86 and HLA-DR expression and an increase in CD14 expression. The constitutively expressed hsc70 had neither a stimulatory effect on the maturation of immature DC nor did it reduce the differentiation of monocytes into DC. These findings demonstrate the specific ability of rhsp70 to induce the maturation of immature DC. Therefore rhsp70 may be useful for its adjuvant like properties in DC based immunotherapy of certain tumors.

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1 Introduction

Cells respond to stress factors such as heat, hypoxia or viral transformation by the synthesis of a group of proteins called heat shock proteins (hsp) [1]. Members of the Hsp70 group are either constitutively expressed (hsc70) or can be induced (hsp70) by stress factors [2]. They function as molecular chaperones for antigenic peptides in the endoplasmic reticulum and cytoplasm and are involved in antigen processing and presentation [3]. As previously shown by our group Hsp70 is also expressed on the surface of human tumor cells such as sarcomas, lung carcinoma and colon carcinoma [4–7] and can act as a recognition structure for NK cells [8]. It has been proposed that hsp can also activate the innate immune response by acting as danger signals [9–10] since hsp70 [11] and hsp60 [9] can directly induce the production of cytokines from monocytes and macrophages. Danger signals [12] are thought to be recognized by pattern recognition receptors on antigen presenting cells (APC) [13–14]. Professional APC such as dendritic cells initiate an immune response after activation and form the link between the innate and an acquired immune response [15]. Dendritic cells are now widely recognized to play an important role in the immune response to tumors [16]. Immature DC specialize in antigen capture and processing whereas mature DC are potent antigen presenting cells [17]. Recently it has been shown that the constitutively expressed hsp gp96, can induce the maturation of dendritic cells derived from $CD14^+$ monocytes [18]

We investigated what effect the heat inducible rhsp70 in comparison to the constitutively expressed rhsc70 had on the differentiation of monocytes into DC in addition to

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Abbreviations: DC: Dendritic cell **Hsp:** Heat shock protein

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analyzing the effect of rhsp 70 and rhsc70 on the maturation of immature DC. We found that rhsp70 in contrast to rhsc70 stimulated the maturation of immature DC but reduced DC maturation when added to monocyte precursor cells. Immature DC were also able to bind rhsp70 whereas minimal binding was observed for mature DC. In addition immature DC stimulated to mature with rhsp70 showed an increased allostimulatory capacity and an enhanced ability to present MHC class I-restricted peptides to specific T cell clones.

2 Results

2.1 Hsp70 induces the maturation of monocyte-derived dendritic cells when added to immature dendritic cells

The phenotype of the starting population of plastic adherent mononuclear cells used to generate DC was characterized by flow cytometry. The cells consisted of on average 70% monocytes. Human rhsp70 (0.5 μg/ml) was added to monocyte-derived DC after 5 days of culture in GM-CSF and IL-4 containing medium and a FACS analysis was done on day 8. Recombinant hsp70 induced DC maturation as shown by an increase in the expression of CD40, CD86 and CD83 molecules in comparison to control cultures (Fig. 1, upper panel). No increase in DC maturation was seen in parallel cultures after the addition of heat denatured (100°C, 20 min) human rhsp70 (0.5 μg/ml) (Fig. 1, lower panel). A comparison was made between the effect of rhsc70 and rhsp70 on DC maturation. When rhsc70 was added, CD83 and CD14 expression were the same as control values (Fig. 2A and B) whereas rhsp70 increased CD83 expression (Fig. 2A) and decreased CD14 expression (Fig. 2B).

When polymyxin B (a potent inhibitor of LPS) was added to cultures it had no inhibitory effect on rhsp70 induced DC maturation but inhibited LPS induced DC maturation (Fig. 3).

2.2 Recombinant hsp70 increases DC allostimulatory capacity and enhances their ability to stimulate specific T cell clones

DC stimulated to mature with rhsp70 (0.5 μg/ml) from days 5 to 8 were incubated with allogeneic lymphocytes for 4 days and cell proliferation was measured. PBL incubated with rhsp70-treated DC showed significantly greater ($p < 0.005$) levels of proliferation in comparison to control cultures with untreated DC (Fig. 4). We also performed a proliferation assay using HLA-A*0201-restricted T cell clones specific for the tyrosinase

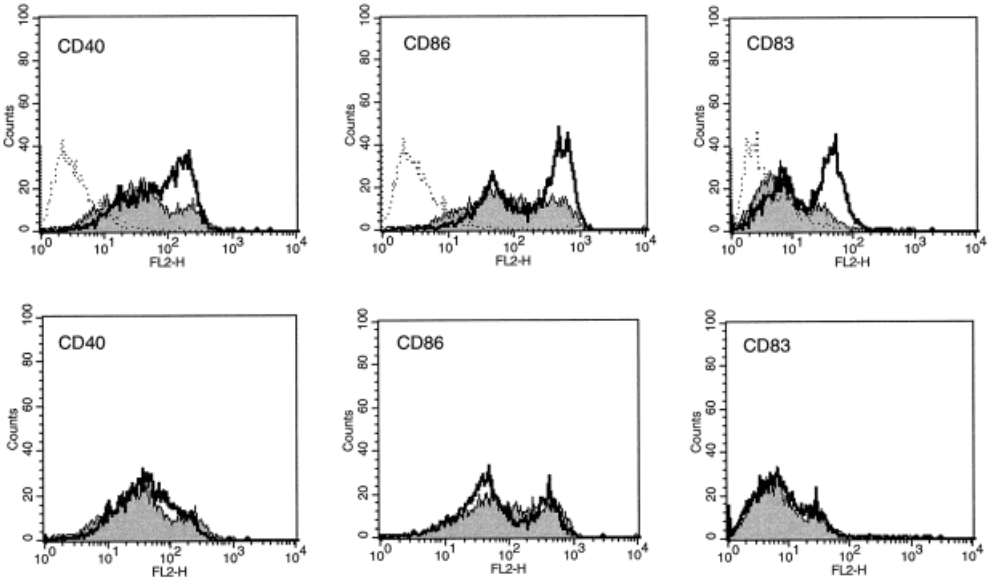

Fig. 1. FACS analysis of CD40, CD86 and CD83 expression in monocyte-derived DC. Monocytes were cultured for 8 days in medium containing GM-CSF and IL-4 alone (gray histogram) or in GM-CSF/IL-4 plus rhsp70 (0.5 μg/ml) added to the cultures on day 5 (black line, upper panel) or in GM-CSF/IL-4 plus rhsp70 (0.5 μg/ml) heat denatured (100°C, 20 min) (black line, lower panel) added to the cultures on day 5. The dotted line represents isotype control antibodies which showed the same fluorescence intensities with the addition of rhsp70 or heat denatured rhsp70. The results are representative of three separate experiments.

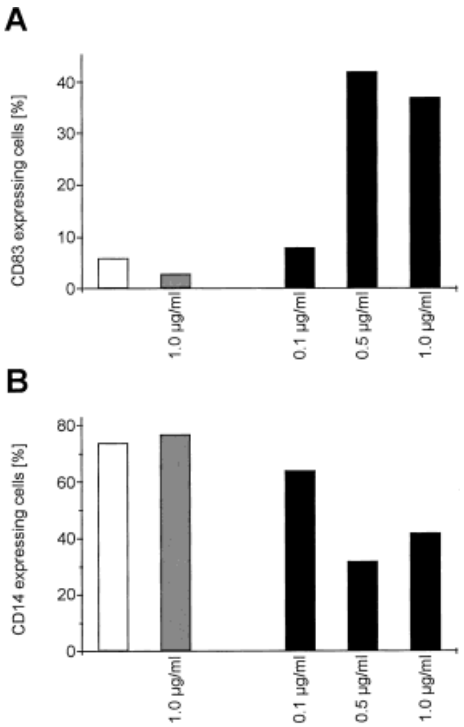

Fig. 2. Comparison of the effect of rhsc 70 and rhp70 on DC maturation. (A) CD83 and (B) CD14 expression on DC cultured for 8 days in medium containing GM-CSF and IL-4 alone (white bar) or in GM-CSF/IL-4 plus rhsc70 (gray bar) added on day 5 or in GM-CSF/IL-4 plus increasing concentrations of rhsp70 (black bars) added on day 5. The results are representative of two separate experiments.

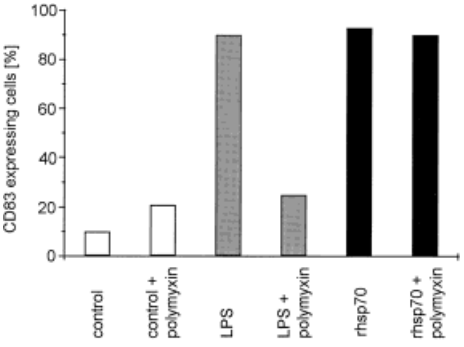

Fig. 3. Effect of polymyxin B on LPS and rhsp70 induced DC maturation. DC were cultured for 8 days in GM-CSF and IL-4 containing medium plus or minus polymyxin (0.5 µgml) added on day 5 (white bars) or with LPS (100 ng/ml) plus or minus polymyxin added on day 5 (gray bars) or with rhsp70 (0.5 µg/ml) plus or minus polymyxin added on day 5 (black bars). A FACS analysis of CD83 expression was done on day 8. The results are representative of two separate experiments.

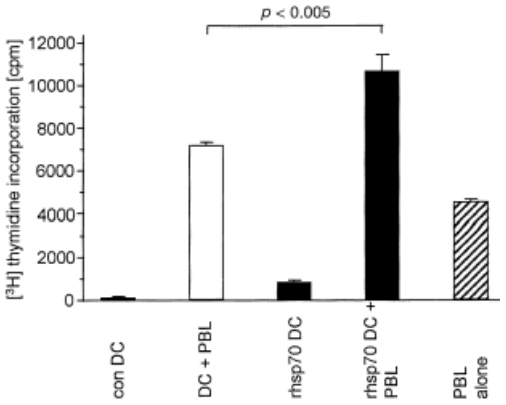

Fig. 4. Recombinant hsp70 enhances DC allostimulatory capacity. DC were cultured for 8 days in medium containing GM-CSF and IL-4 with or without rhsp70 added on day 5. The DC were then washed and allogeneic PBL (10^5)/well were added to untreated DC (DC + PBL) or rhsp70 treated DC (rhsp70 DC + PBL) for 4 days at different stimulator/responder ratios (the results show ratio 1:10). T cell proliferation was assessed by measuring the uptake of [^{3}H]thymidine (1 µCi/well) during the last 24 h of culture. Control cultures with rhsp70 stimulated DC alone (rhsp70 DC) or untreated DC (DC) alone or PBL alone were also included. DC treated with rhsp70 show a significant ($p<0.005$) increase in allostimulatory capacity. The results are the mean cpm and SD of triplicate samples. One experiment representative of three is shown.

369–377 nonapeptide, a known HLA-A*0201-restricted CTL epitope. DC from compatible donors cultured in the presence of rhsp70 (0.5 µg/ml) from days 5 to 8 showed a significantly ($p< 0.05$) enhanced ability to stimulate tyrosinase specific T cell clones when pulsed with low concentrations (1 µg/ml) of tyrosinase peptide in comparison to peptide-pulsed DC cultured in GM-CSF and IL-4 alone (Fig. 5A). In parallel cultures set up to measure IFN-γ production from the CTL clone, the DC cultured in the presence of rhsp70 (0.5 µg/ml) from days 5 to 8 and pulsed with tyrosinase peptide also showed an increased ability to stimulate IFN-γ production from tyrosinase specific CTL (Fig. 5B). No IFN–γ production was obtained from DC or CTL alone. Very low levels of IFN–γ production were observed in DC /CTL cultures in which the DC had not been pulsed with tyrosinase peptide (Fig. 5B).

2.3 Hsp70 reduces the level of DC maturation when added to monocytes at the initiation of culture

Although rhsp70 could induce DC maturation when added to immature DC we found it had the opposite effect when added to monocyte cultures.

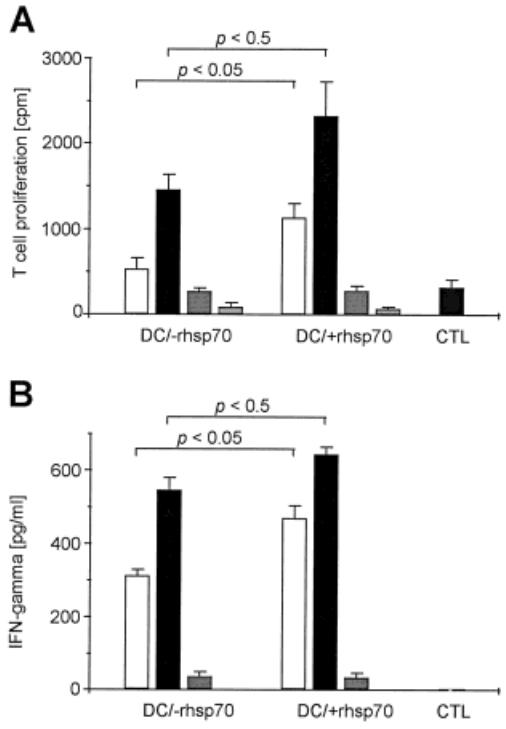

Fig. 5. Antigen-specific T cell stimulation. DC were cultured for 8 days in medium containing GM-CSF and IL-4 alone (DC-rhsp) or with rhsp70 (0.5 μg/ml) added on day 5 of culture (DC + rhsp). DC were then plated at 10^4/well in 100 μl medium in 96-well round-bottom plates and pulsed with tyrosinase peptide 1 μg/ml (white bar) or 10 μg/ml (black bar) for 2 h and then irradiated. Tyrosinase-specific CTL (2×10^4) were then added in a final volume of 100 μl to each well in medium containing 20% FCS and 100 U/ml IL-2. Control cultures containing non- tyrosinase pulsed DC plus CTL (dark gray bars) or DC alone (light gray bars) or CTL alone (CTL) were also included. (A) The cells were cultured for 72 h and [^{3}H]thymidine was added for the last 24 h of culture. The results are the mean values of triplicate cultures ± standard deviation. The results are representative of three separate experiments. Recombinant hsp70 treated DC pulsed with 1 μg/ml of tyrosinase peptide showed a significantly ($p<0.05$) increased ability to stimulate CTL proliferation. (B) Cultures were set up in parallel for IFN–γ production. Supernatants (100 μl) were removed from each well after 24 h and assayed for IFN–γ production using an ELISA kit specific for IFN–γ. The results are the mean values of triplicate cultures ± standard deviation. The results are representative of three separate experiments. Recombinant hsp70 treated DC pulsed with 1 μg/ml of tyrosinase peptide showed an increased ($p<0.05$) ability to stimulate IFN–γ production from tyrosinase specific CTL.

Human rhsp70 (0.1–1 μg/ml) was added at the same time as GM-CSF and IL-4 to adherent monocytes at the initiation of culture. The DC generated after 8 days showed reduced levels of maturation in comparison to control cultures when rhsp70 was added to cultures on day 0. FACS analysis showed a decrease in the expression of CD1a, CD40, CD83, CD86 and HLA-DR molecules and an increase in the expression of CD14 in comparison to control cultures (Fig. 6A). The inhibitory effect was concentration dependent with the maximum effect being found between 0.5 and 0.7 μg/ml of rhsp70 (Fig. 6B). High or moderate CD83 expression could be found in some control cultures after 8 days but in each case the presence of rhsp70 from day 0 caused a reduction in the number of CD83-positive cells (results not shown). Heat denatured rhsp70 (100°C for 20 min) and rhsc70 were also added at the initiation of culture at the same concentration as rhsp70 (0.5 μg/ml) but had no inhibitory effect on DC generation (results not shown).

2.4 Monocytes, immature DC and mature DC differ in their ability to bind rhsp70

Monocytes obtained after a 2 h adherence to plastic were analyzed for their ability to bind rhsp70. A moderate number of $CD14^+$ cells bound rhsp70 (Fig. 7A). When monocyte-derived DC grown for 8 days in medium containing GM-CSF and IL-4 were incubated with FITC-labeled rhsp70 immature DC expressing either low levels of CD83 or no CD83 bound rhsp70 to a greater extent (Fig. 7B) than monocytes (Fig. 7A). When DC were stimulated to mature by adding a cytokine maturation cocktail (containing IL1-β, IL-6, TNF-α, PGE2) to cultures on day 6 to day 8, DC expressing high levels of CD83 (mature) showed minimal binding of rhsp70 (Fig. 7C). FITC-labeled BSA did not bind to either immature or mature DC populations (Fig. 7B and C).

3 Concluding remarks

Our results show the specific ability of rhsp70 to induce the maturation of immature (differentiated) DC. However, the opposite effect is found when hsp70 is added to monocytes (differentiating precursors) at the same time as GM-CSF and IL-4 in that DC maturation is reduced. These effects were found only with rhsp70 and not with rhsc70 or heat denatured rhsp70. We also show that immature DC could bind rhsp70 whereas mature DC could not. Functional studies also revealed that immature DC stimulated with rhsp70 showed an increased allostimulatory capacity and enhanced ability to present peptides to specific T cell clones in comparison to DC cultured in GM-CSF and IL-4 alone.

It has been reported that exogenous hsp70 can bind to the surface CD14 receptor of human monocytes with subsequent up-regulation in the expression of pro-

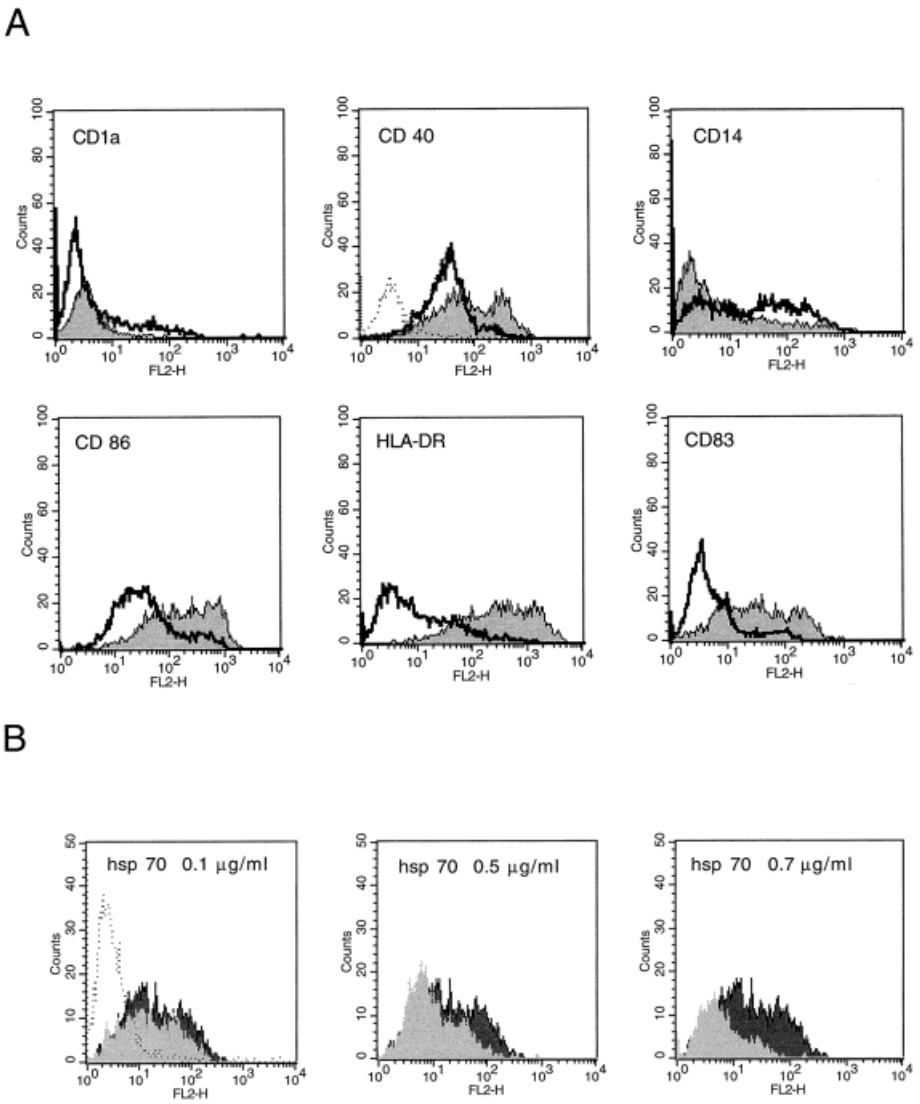

Fig. 6 (A) Phenotypic analysis of monocyte-derived DC cultured for 8 days in medium containing GM-CSF and IL-4 alone (gray histogram) or in GM-CSF/IL-4 plus rhsp70 (0.5 μg/ml) (black line). The dotted line represents an isotype control antibody. The isotype controls showed similar levels of fluorescence for all the markers studied. The results are representative of three separate experiments. (B) CD83 expression in monocyte-derived DC cultured for 8 days in medium containing GM-CSF and IL-4 alone (black histogram) or in GM-CSF/IL-4 plus increasing concentrations of rhsp70 (0.1–0.7 μg/ml) (gray histogram).The dotted line represents the isotype control fluorescence. The results are representative of three separate experiments.

inflammatory cytokines such as TNF-α, IL-6 and IL-1β [11]. A combination of these cytokines plus PGE2 has been used to induce the maturation of immature DC for immunotherapeutic purposes [19]. If monocytes in the presence of GM-CSF and IL-4 could be triggered directly by hsp70 induced cytokines to differentiate into mature DC this, as has been previously suggested [20], would not be the most efficient mechanism for inducing immunity since immature DC need to capture and process antigens. It was found [20] that the presence of hsp70 in tumor cell lysates could target immature DC precursors and maintain the DC population in a more poorly differentiated state. With respect to monocyte precursors as we have shown, the presence of hsp70 reduces the maturation of dendritic cells, however, it may be that in addition to stimulating the production of IL-1β, IL-6 and TNF-α from monocytes [11], hsp70 stimulates the production of other inflammatory cytokines such as M-CSF which would shift the balance more in the direction of monocytes [21].The study of Todryk [20] also found that once the DC had reached a certain level of maturation they were no longer responsive to the inhibitory effect of

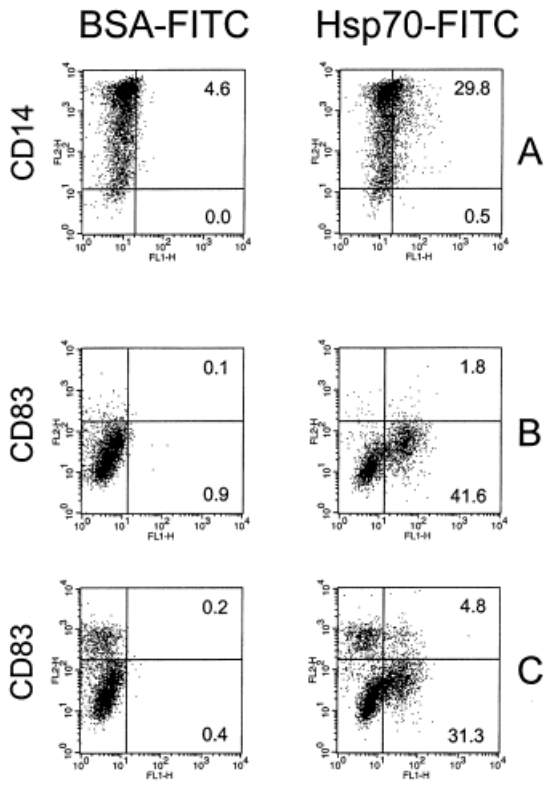

Fig. 7. (A) Freshly isolated monocytes or (B) monocyte-derived DC obtained after 8 days in culture in medium containing GM-CSF and IL-4 alone or (C) monocyte-derived DC cultured in GM-CSF/IL-4 containing medium with a cytokine maturation cocktail added from day 6 to 8 were stained for CD14 or CD83 and PE labeled. Cells were also incubated with FITC-conjugated BSA or FITC-conjugated rhsp70. FITC-conjugated rhsp70 bound to CD 14 + monocytes and to immature DC (CD83 low or negative) but not mature DC (CD83 high) None of the cells bound FITC-conjugated BSA. Results are representative of three separate experiments.

hsp70. Our own results have shown that immature DC can bind and be stimulated to mature by rhsp70. In contrast, mature DC no longer bind rhsp70 which may be due to a down-regulation of the receptor for hsp70.

Another stress protein, gp96, can induce DC maturation [18] and the binding of gp96 by its receptor, recently characterized as CD91 [22] is also down-regulated in mature DC [18]. Thus immature DC that can bind specific heat shock proteins such as hsp70 and gp96 are more likely to be able to capture and process antigens whereas mature DC that have lost the ability to bind heat shock proteins would be better at antigen presentation.

Dendritic cells can also deliver Ag directly after incubation with preprocessed synthetic peptide to class I restricted cytotoxic T cells [23]. DC pulsed with tumor-derived peptides have been used in immunotherapy trials of certain tumors such as melanoma [24]. We found that immature DC treated with rhsp70 were more efficient in presenting tyrosinase peptide to specific CTL clones. Thus immature DC stimulated to mature with rhsp70 and then pulsed with tumor peptides may also be of use in enhancing a tumor specific immune response. Recently it was shown that immunization of mice with DC pulsed with tumor derived hsp110 resulted in the inhibition of tumor growth [25] which suggests that DC are involved in hsp-induced antitumor responses. Recombinant hsp70 can enhance cytokine production from monocytes [11], and also enhances NK cell proliferation and cytotoxicity whereas hsc70 does not [26], therefore it would appear that rhsp70 can enhance both specific and innate immune responses. Hsp70 may act as a danger signal that is recognized by both DC and NK cells thus inducing the activation of both the adaptive and innate immune responses and promoting cross talk [27] between the two systems. Induction of hsp70 on tumors *in vivo* by hyperthermia may also provide a danger signal to the immune system that promotes an anti tumor response *in vivo* ([28].

4 Materials and methods

4.1 Generation of dendritic cells

Peripheral blood mononuclear cells (PBMC) were prepared from leukapheresis samples by density gradient centrifugation over Ficoll/hypaque (Pharmacia, Biotech, Freiburg, Germany). To obtain $CD14^+$ monocytes, 30×10^6 PBMC were incubated in $75cm^2$ plastic flasks (Nunc, Wiesbaden, Germany) for 2 h and the nonadherent cells washed off. The adherent cells were then cultured for 8 days in RPMI VLE (Biochrom, Berlin, Germany) supplemented with 2 mM glutamine, 100 U/ml pen/strep (all from Life Technologies, Karlsruhe, Germany) and 1% autologous serum. To generate DC, GM-CSF (500 U/ml) (Hölzel Diagnostika, Köln, Germany) and IL-4 (800 U/ml) (Biomol, Hamburg, Germany) were added on day 0 and GM-CSF was added again on day 4 of culture.

4.2 Stimulation of monocytes and immature dendritic cells

Human recombinant hsp70 (0.1–1 μg/ml) (StressGen Biotechnologies, Victoria, Canada) was added to monocytes on the same day as the addition of GM-CSF and IL-4, day 0, (*i.e.* to differentiating precursors) or after the monocytes had been cultured in GM-CSF and IL-4 for 5 days (*i.e.* to differentiated DC). A FACS analysis of cell surface markers was done on day 8. Control cultures were set up in medium plus GM-CSF and IL-4 alone or with the addition of human

recombinant hsc70 (0.5-1 μg/ml) (StressGen Biotechnologies, Victoria, Canada) or human recombinant hsp70 (0.5–1 μg/ml) heat denatured (100°C for 20 min) either at the initiation of culture (day 0) or on day 5 of culture. Parallel control cultures containing polymyxin B (0.5 μg/ml) (Sigma, Deisenhofen, Germany) were also included.

Hsp70 (0.5 μg/ml) or LPS (100 ng/ml)(Sigma) ± polymyxin B (0.5 μg/ml) were also added to dendritic cells on day 5 and a comparison made of their effects on dendritic cell maturation on day 8.

4.3 FACS analysis

The antibodies used to assess DC maturation by FACS analysis included CD1a, CD40, CD86 (Pharmingen, Hamburg, Germany), CD14 and CD83 (Immunotech, Hamburg Germany) and HLA-DR. The isotype controls used included IgG1, IgG2a and IgG2b (all from Immunotech). Cells were washed in PBS containing 5% FCS. Staining was performed at 4°C for 30 min using mouse mAb to the markers mentioned above. The cells were then washed and incubated with PE-conjugated goat anti-mouse IgG (Dako, Hamburg, Germany) for 30 min at 4°C. The cells were then washed and resuspended in 500 μl of PBS (Life Technologies) plus 5% FCS (Biochrom). All FACS analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA) using Cell Quest Software.

4.4 Mixed leukocyte reactions (MLR).

DC were cultured as above with or without rhsp70 (0.5 μg/ml) added from day 5 to 8.The DC were then washed and resuspended in RPMI medium plus 10% FCS and incubated with allogeneic PBMC (10^5/ well) in 96-well plates at different responder to stimulator ratios for 4 days. Control wells contained either rhsp70 treated DC or untreated DC alone or PBMC alone. T cell proliferation was assessed by measuring the uptake of [^{3}H]thymidine (1 μCi/well) (Amersham, Pharmacia Biotech, Freiburg, Germany) during the last 24 h of culture using a microBeta counter (Beckman, Germany).

4.5 T cell proliferation assay and IFN–γ production

Monoyctes were isolated from an HLA-A*0201 donor and immature DC generated as described above. Hsp70 (0.5 μg/ml) was added to immature DC from days 5 to 8 of culture. The cells were then harvested and resuspended at 10^4 DC/well in 100 μl of medium in 96-well round bottomed plates (Nunc). The DC were then pulsed with tyrosinase 369–377 peptide (1–10 μg/ml) for 2 h and irradiated. Tyrosinase peptide-specific T cells (2×10^4) in 100 μl RPMI medium (Biochrom) containing 10% FCS and 100 U/ml IL-2 (Biomol) were then added to each well. Control wells contained non-tyrosinase pulsed DC and CTL or DC alone or CTL alone. Cells were incubated for 72 h at 37°C and 1 μCi of [^{3}H]thymidine was added to the wells for the last 24 h of culture. The amount of [^{3}H]thymidine incorporated was detected using a microBeta counter. Parallel cultures were also set up and the supernatants (100 μl) removed after 24 h of culture. The amount of IFN–γ produced was determined using an IFN–γ-specific ELISA kit (cytimmune, Maryland).

4.6 FITC labeling of rhsp70

Recombinant hsp70 (Stressgene) and BSA fraction V (Sigma) were incubated with FITC (Sigma) in 0.1 M carbonate-bicarbonate buffer overnight at 4°C with gentle agitation. Free FITC and low molecular reaction by-products were removed by separating the mixture by gel filtration utilizing Sephadex G-25. Fractions containing protein were collected. The number of FITC molecules was estimated to be between 3 to 4 per molecule of protein by comparison of the optical densities at 280, 495, and 490 nm. The conjugated proteins were tested for identity by SDS-PAGE and immuno blotting with the respective specific antibodies against hsp70 (SPA810, Stressgene), and with anti-FITC mAb (Dako, Hamburg, Germany).

4.7 FACS analysis of binding of FITC-labeled rhsp70 to monocytes, immature DC and mature DC

Monocytes were obtained after a 2 h adherence of PBMC to plastic. The nonadherent cells were washed off and the adherent cells collected. Immature DC were generated by culturing monocyte precursor cells in GM-CSF and IL-4 containing medium for 8 days as described in section 4.1. Mature DC were also obtained by adding a maturation cocktail containing IL-1β, IL-6, TNF-α and PGE2 [19] to DC on day 6 to day 8. Cells were stained for CD14 and CD83 and PE labeled as described in section 4.3. The cells were then incubated with the FITC-conjugated proteins for 30 min on ice in medium containing 1% autologous serum at a concentration of 10 μg/ml. After washing the cells were fixed with paraformaldehyde and analyzed by flow cytometry. Cells were also labeled with propidium iodide and positive cells were gated out.

4.8 Statistical analysis

Data are expressed as the mean values ± SD of triplicate samples. The statistical significance of the differences was determined by the unpaired two tailed Student's *t*-test. Differences were considered statistically significant for $p < 0.05$.

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