

Activation of NK cells by extracellular heat shock protein 70 through induction of NKG2D ligands on dendritic cells

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We have previously reported that heat shock protein 70 (HSP70) vaccination induced natural killer (NK) cell activity in patients with chronic myelogenous leukemia (CML). In this study, HSP70 of both autologous and allogeneic origin was found to stimulate IFN- γ production from peripheral blood mononuclear cells of CML patients, as well as of normal subjects. Further investigations demonstrated that the activity of HSP70 was dependent on both NK cells and dendritic cells (DCs). HSP70 did not induce significant IFN- γ production from either NK cells or DCs alone. Mechanistically, we found that HSP70-mediated DC-NK cell crosstalk required cell-cell contact, which could be inhibited completely by neutralizing antibody against NK activating receptor NKG2D. The significance of NKG2D was further corroborated by the finding that HSP70 induced the expression of an NKG2D ligand, the MHC class I chain-related protein A (MICA), on DCs; HSP70-augmented IFN- γ release was abrogated by antibody against MICA. Thus extracellular HSP70, released during either stress or inflammatory cell death, may serve as a critical link between NK and DCs in mounting immune responses against infections, cancers and self-antigens.

Keywords: human, chronic myelogenous leukemia, immunization, heat shock protein 70, cellular immunity

Introduction

DCs and NK cells are considered to be critical cellular players of the innate immune system (1, 2). DCs are the most powerful professional antigen presenting cells (APCs) that are instrumental in processing antigens and orchestrating antigen-specific adaptive immunity and tolerance (3-5). In response to viral infections or cellular transformation, NK cells exert cytolytic functions as well as secrete numerous proinflammatory cytokines, both of which are regulated by the delicate balance between inhibitory and stimulatory receptors on NK cells (2, 6). For instance, tumor cells may down-regulate class I molecules of the major histocompatibility complexes (MHC) on cell surfaces to evade CD8+ T cell mediated immune surveillance. However, loss of MHC class I could trigger NK cell recognition due to the removal of negative signals delivered through killer inhibitory receptors on NK cells (2). At the same time, as a result of cellular stress, tumor cells may also express stress-inducible NKG2D ligands resulting in the activation of NK cells through the engagement of the stimulatory receptor NKG2D on NK cells (7).

NK cells and DCs can functionally interact with each other both *in vitro* and *in vivo* (8-10). It was observed initially that

DC-mediated eradication of MHC class I-negative tumors was dependent on NK cells (11). It was later found that NK cells could activate DCs in both a contact-dependent and a TNF- α /IFN- γ -dependent manner (11-13). Reciprocally, DCs are armed with the ability to activate resting NK cells (11, 12, 14). NKp30 was shown to be partially responsible for DC-mediated NK cell activation (13, 14). In addition, both IL-12 (15) and NKG2D ligands (16) on DCs have been implicated in the cross-activation of DCs and NK cells. However, the underlying mechanism by which DC-NK cell crosstalk is initiated remains enigmatic (10).

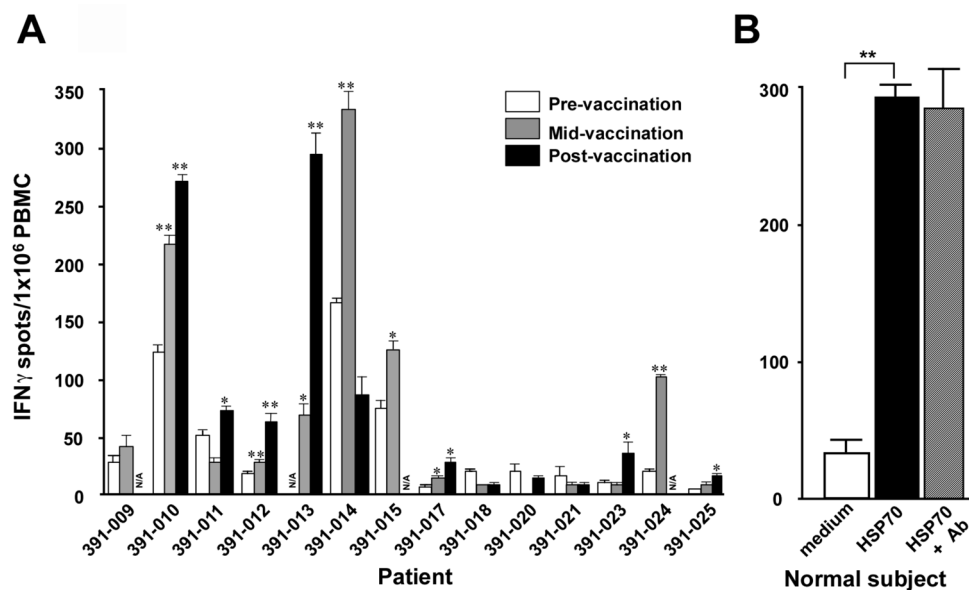
Heat shock proteins (HSPs) are intracellular molecular chaperones that play essential roles in facilitating protein folding (17). Soluble HSPs have also emerged as key host-derived regulators of the immune system largely because of their ability to interact with APCs and to chaperone antigenic peptides for cross-presentation to MHC class I and class II molecules on APCs (18). Indeed, tumor-derived HSP-peptide complexes have entered into various phases of clinical trials against cancer (19-21). We have previously demonstrated that vaccination with HSP70 was associated with increased T cell, as well as NK cell, activity in patients with CML (21). In understanding the mechanism of HSP70 in inducing NK cell activity, we found that HSP70 did not activate NK cells directly. Instead, HSP70 induced the expression of an NKG2D ligand MICA on DCs, which then activated NK cells in an NKG2D-dependent manner. Our study has therefore uncovered another facet of the immunological properties of HSPs in mediating crosstalk between DCs and NK cells, reinforcing the notion that host-derived stress-inducible HSPs may participate in the initiation of immune responses.

Results

Induction of IFN- γ release by HSP70

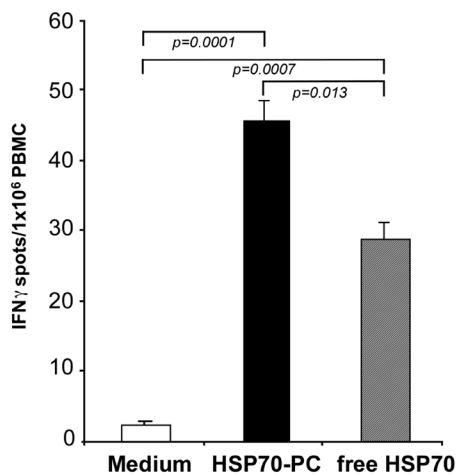
To understand the potential roles of HSP70 in the immunotherapy of leukemia, we have completed and reported the phase I study of autologous HSP70 vaccine in 20 patients with chronic phase CML (21). The clinical grade, endotoxin-free (<0.05 EU/ml as determined by the limulus amoebocyte lysate assay) HSP70 was purified from the autologous leukocytes that were obtained by leukaphoresis. In our study, we serendipitously discovered that autologous HSP70 could stimulate significant IFN- γ production, as measured by an ELISPOT assay, from unfractionated PBMCs from all CML patients (Figure 1A). The magnitude of the IFN- γ response was

Figure 1



Clinical grade HSP70 induces IFN- γ release from PBMCs of CML patients as well as normal subjects. (A) Endotoxin-free HSP70 was incubated with PBMCs from CML patients collected two weeks before the first vaccine (pre), one week after the fourth vaccine (mid) and two weeks after the last vaccine (post) for the quantification of the frequency of IFN- γ producing cells by ELISPOT. N/A represents data points not available due to insufficient cells. *P* values: *, <0.05, **, <0.005. (B) Frequency of IFN- γ producing cells from PBMCs of normal subjects stimulated with HSP70 in the presence or absence of a pan-HLA class I blocking Ab W6/32 (Ab).

Figure 2



Induction of IFN- γ release from PBMCs by peptide-free HSP70. IFN- γ ELISPOT assays were performed as in Figure 1 except that PBMCs were stimulated with different forms of HSP70, including autologous HSP70-peptide complex (HSP70-PC) and free HSP70 stripped of peptides by ATP. *P* values are indicated.

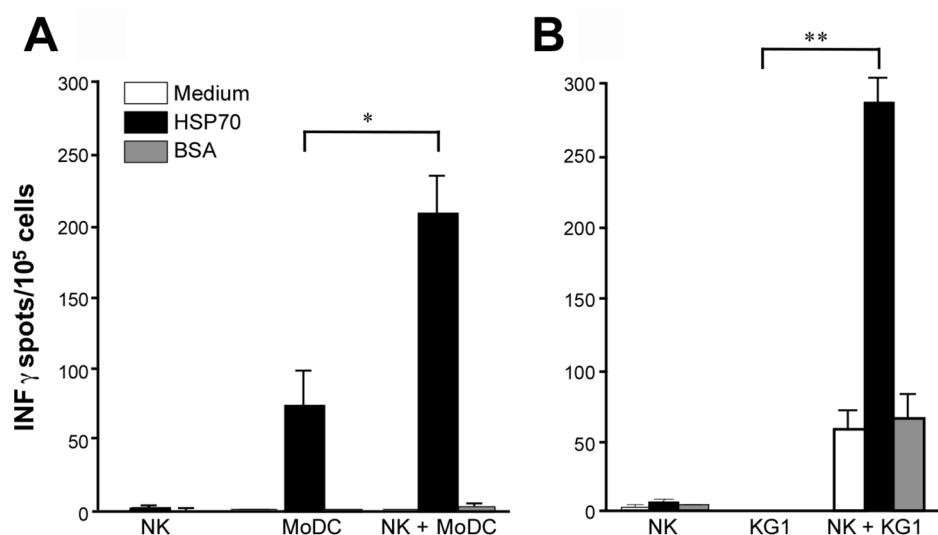
different from patient to patient and correlated with the number of functional NK cells, as measured by IFN- γ ELISPOT against K562 cells, the standard targets of NK cells (21). In addition, 10 out of 14 patients had significantly increased IFN- γ producing cells in the peripheral blood after HSP70 vaccinations, which is again in line with increased NK cell activity as reported in our original study in these patients (21).

We also stimulated PBMCs from normal subjects with allogeneic HSP70 and observed the presence of a considerable number of HSP70-inducible IFN- γ producing cells in the peripheral blood (Figure 1B). IFN- γ release from PBMCs in response to HSP70 was additionally observed in patients with ovarian cancer (data not shown), demonstrating that the ability of HSP70 to modulate IFN- γ release from PBMCs is not uniquely restricted to patients with CML.

HSP70 is known to chaperone cellular peptides from a diverse array of cellular proteins (22, 23). The composition of peptides associated with HSP70 depends on the cells or tissues from which HSP70 is isolated from. The fact that PBMCs from normal subjects responded to patient's leukocyte-derived HSP70 argues against CML-specific peptides in the HSP70 preparation contributing to the observed activity. Indeed the IFN- γ release from normal PBMCs in response to CML HSP70 could not be blocked by a pan-HLA class I-specific mAb, W6/32 (Figure 1B), which was effective in blocking CD8+ T cell-mediated activity completely (data not shown). To further differentiate the contribution by HSP70 protein from HSP70-associated peptides of other proteins, we stripped off peptides from HSP70-peptide complexes (HSP70-PC) by the standard adenosine triphosphate (ATP) treatment and size exclusion centrifugation (22). In this case, ATP hydrolysis induces conformational change of HSP70, leading to the dissociation of HSP70 from the peptides from the substrate-binding pocket of HSP70. We found that both HSP70-PC and peptide-free HSP70 were able to substantially induce IFN- γ release from human PBMCs (Figure 2); removal of HSP70-associated peptides only resulted in a partial loss of IFN- γ activity.

Our data suggests that the immunological activity of HSP70 illustrated in Figure 1 and Figure 2 resides to a large extent in the HSP70 itself, but not in the peptides chaperoned by HSP70. The major IFN- γ producing cells in the peripheral blood include T

Figure 3



Co-culture of HSP70 with DCs and NK cells, but not with NK cells or DCs alone, leads to a significant increase in IFN- γ producing cells. CD56+ cells were cultured alone, or with either MoDCs (A) or KG1 (B), in the presence of medium control, HSP70 or BSA for 48 hours. The frequency of IFN- γ producing cells was determined by ELISPOT. *P* values: *, <0.05, **, <0.005. More than 10 independent experiments were performed with similar findings.

lymphocytes (CD4+ and CD8+ T cells) and NK cells. The experiments so far suggest that a contribution by T cells is unlikely because of the fact that "unprimed" normal subjects respond to HSP70 stimulation in an MHC class I-unrestricted manner (Figure 1B) and that the IFN- γ release elicited by HSP70 is mostly independent of peptides (Figure 2).

DC-NK cell interaction

We next focused our attention on NK cells based upon the above considerations. We were also partly motivated by a study demonstrating the possible properties of HSP70 to directly activate NK cells (24). To examine this possibility, we enriched CD56+ NK cells from the PBMCs using magnetic beads and co-cultured these highly purified NK cells with HSP70 (over 90% purity by CD56 cell surface marker; data not shown). However, we found that HSP70 alone was unable to directly activate NK cells from either normal subjects or CML patients to elicit IFN- γ production (Figure 3). This data however could not rule out the possibility that there was another cell type in the PBMCs that might indirectly trigger NK cell activation in response to HSP70. Given the appreciation of the ability of DCs to activate NK cells, we examined the possibility of professional APCs, DCs in particular, in potentiating HSP70-induced IFN- γ production from NK cells. We obtained DCs from two sources: autologous peripheral blood monocyte-derived DCs (MoDCs) and an established allogeneic DC-like cell line, KG1 (25). We found that the co-culture of NK cells and DCs, alone or in the presence of a control protein BSA resuspended in the same buffer as HSP70, resulted in no significant production of IFN- γ (Figure 3). However, stimulation by HSP70 resulted in a marked increase in the number of IFN- γ producing cells when NK cells were co-cultured with either MoDCs (Figure 3A) or KG1 (Figure 3B). This experiment strongly suggests that NK cells and DCs are responsible for HSP70-mediated production of IFN- γ .

Roles of NKG2D

To understand the molecular mechanism by which HSP70 promotes DC-NK cell interactions, we performed a sequential

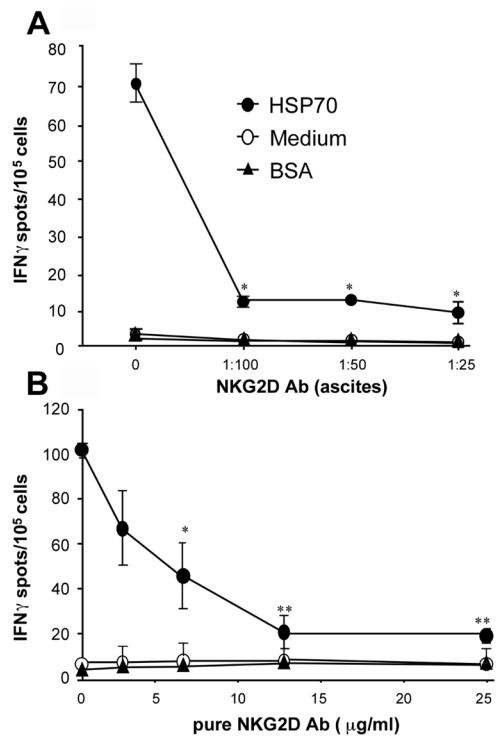
experiment by incubating NK cells with either HSP70-treated DCs or supernatant from these DCs. We found that the increased IFN- γ activity can only be reconstituted by DCs but not by condition medium from HSP70-treated DCs (data not shown), suggesting that direct DC-NK cell interaction is required for NK cell activation by HSP70.

The activation of NK cells by DCs has been shown to be mediated in part by the stimulation of the activation receptor NKG2D on NK cells by NKG2D ligands (NKG2DL) expressed on DCs (7). In the murine models, for example, it was found that the stimulation of Toll-like receptors (TLRs) on DCs could result in the induction of an NKG2DL Rae1 (16). We therefore postulated that HSP70 induces the expression of NKG2DL on DCs, which then activates NK cells in an NKG2D-dependent manner. To test this possibility, we co-cultured DCs, NK cells and HSP70 in the presence of an antagonistic mAb against NKG2D, 1D11 (26). As demonstrated in Figure 4, 1D11 blocked HSP70-stimulated IFN- γ production in a dose-dependent manner regardless of what DCs (MoDCs or KG1) were used (Figure 4, panels A and B). An isotype control Ab did not block IFN- γ release (data not shown). Since the blockage by anti-NKG2D Ab is nearly complete, we conclude that the NKG2D pathway is the key mechanism by which HSP70 induces IFN- γ production from NK cells in our system.

Roles of MHC class I chain-related protein A

There are at least six NKG2DLs in humans, including UL16-binding protein (ULBP) 1, ULBP2, ULBP3, ULBP4, MICA and MICB, some of which have been found to be expressed in DCs (7). To determine the possibility that HSP70 induces the expression of NKG2DL on DCs, we performed a quantitative polymerase chain reaction (qPCR) to measure the amount of mRNA of all known NKG2DLs in HSP70-treated MoDCs. Only *MICA* and *MICB* mRNA levels were induced by HSP70 (data not shown). To determine if MICA protein was expressed after HSP70 treatment, we incubated MoDCs with HSP70 or BSA in the identical buffer followed by flow cytometric analysis for cell surface MICA. We demonstrated that CD11c+ MoDCs indeed

Figure 4



HSP70-mediated crosstalk between NK cells and DCs is dependent on NKG2D. CD56⁺ cells were cultured with either MoDCs (A) or KG1 (B) in the presence of a blocking Ab against NKG2D or an isotype control Ab. The frequency of IFN- γ producing cells was determined by ELISPOT. P values: *, <0.05, **, <0.005. This experiment was repeated twice with similar findings.

express surface MICA after treatment with HSP70, but not with BSA (Figure 5A). NK cells do not express either MICA protein by flow cytometry or *MICA* mRNA by qPCR (data not shown).

To understand the functional significance of MICA expression on the DCs, we introduced an antagonistic mAb against either MICA/MICB (6D4) or MICA (2C10) in the IFN- γ induction assay. We found that the induction of IFN- γ by HSP70 was abrogated by treatment with either of these mAbs, but not by an isotype control Ab (Figure 5, panels B and C). This data, together with the blocking effect of anti-NKG2D Ab (Figure 4), confirmed the crucial roles of the NKG2D-MICA interaction in HSP70-mediated DC-NK cell interactions. The dependence on MICA for activation strongly suggests that the effect of HSP70 is not mediated directly by its potential interaction with NKG2D (27).

Discussion

DCs and NK cells, two of the critical players of the innate immune system, have been lifted to the central stage of immunology due to their instrumental roles in regulating adaptive immunity. For example, it is now believed that DCs are the only cell type that can efficiently prime T cell immunity *in vivo* (28-32), whereas NK cells have been discovered to be one of the key regulators of T cell responses due to their ability to rapidly produce type I proinflammatory cytokines, including IFN- γ and IL-12 (33). It was therefore especially exciting when it was realized that DCs and NK cells are able to cross-activate

each other. Understanding the molecular mechanism by which DC-NK cell interaction is regulated should have fundamental implications in immunology. In this study, we have discovered a positive switch of this regulation. We found that soluble HSP70, which might be released as a result of stress and cell death *in vivo* (34), can potentiate DC-NK cell interaction, leading to an amplified IFN- γ response (Figure 3). HSP70 does so by inducing the expression of NKG2DL MICA on DCs (Figure 5).

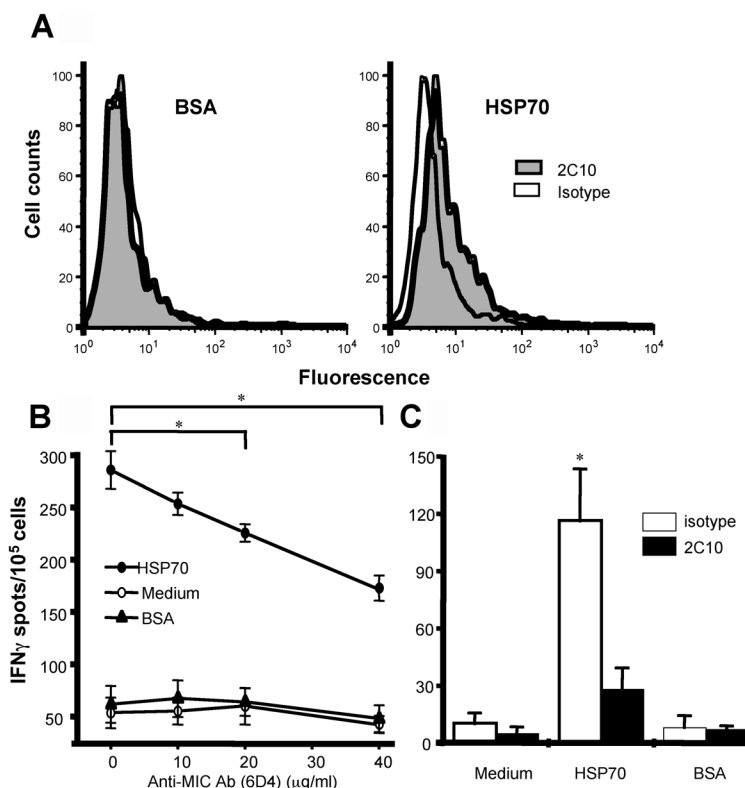
Our report is reminiscent of a study showing that another HSP, gp96, was able to trigger NK cell activity by inducing the expression of NKG2D on NK cells *in vitro* (35). In a murine model, it was demonstrated that gp96-mediated CD8⁺ T cell priming was preceded by, and dependent on, NK cell activation (36). Furthermore, Multhoff and colleagues have reported the direct activation of NK cell lytic functions by HSP70 (24). It was also shown that HSP70 can potentially engage NKG2D directly for its action (27). These studies, along with our present findings, strongly indicate that one of the important properties of extracellular HSPs is to regulate the function of NK cells. It is conceivable that the release of HSPs, as a result of pathological cell stress and death, not only activates DCs for the priming of antigen-specific T cells, but also sends a wave of signals to activate NK cells for the immediate containment of damages from pathological conditions.

It is worthwhile to mention that in our studies we did not find a significant induction of NKG2D on NK cells by HSP70 (data not shown). This is in contrast to the finding that the induction of NK cell-like activity by gp96 is through upregulation of NKG2D on NK cells (35). These findings would suggest that there are differences in the immunological activities amongst the many members of the HSP family. Understanding the immunological heterogeneity of HSPs should be a fruitful area of future research.

IFN- γ production by PBMCs as a result of HSP70 treatment has been observed in CML patients, normal subjects (Figure 1), as well as in patients with ovarian cancer (data not shown). The magnitude of IFN- γ release is heterogeneous, although the percentage of CD56⁺ NK cells in the PBMCs of subjects in this study did not vary significantly (data not shown). This finding is again consistent with our previous work, demonstrating by IFN- γ ELISPOT against K562 cells, that normal subjects have 340 functional NK cells in 1×10^6 PBMCs (range 154-580; $n = 11$). In contrast, CML patients had significantly decreased NK activity, with pre-vaccination NK cells of only 16 to 107 per 1×10^6 PBMCs (21). The functional heterogeneity of the NK response to HSP70 could be due to many reasons, which is the future focus of our study. For example, MICA is an extremely polymorphic molecule. It is unclear at this point if different alleles of MICA interact with NKG2D with different affinity and thus transmit a different strength of downstream signals. Moreover, the lack of stability of MICA on the cell surface in some patients might also lead to clipping of MICA and the generation of soluble MICA, resulting in dampening of NKG2D signaling (37). Additionally, the ability of HSP70 to regulate DC function is likely dependent on the functional integrity of receptors such as CD91 on DCs (38). It has recently been shown that the level of CD91 expression can be subject to modulation in a number of pathological conditions (39). Further studies in addressing these possibilities shall undoubtedly shed light on the mechanisms of the complex interactions between HSP70, DCs and NK cells.

Although *MICB* can also be transcriptionally induced by HSP70 (data not shown), MICA seems to play more dominant roles in mediating DC-NK cell interaction in response to

Figure 5



Roles of MICA in HSP70-mediated crosstalk between NK cells and DCs. (A) MoDCs were treated with HSP70 or BSA, followed by flow cytometric analysis for cell surface MICA expression using MICA-specific mAb 2C10. (B and C) CD56⁺ cells were cultured alone or with MoDCs, in the presence of plain medium, HSP70 or BSA along with anti-MIC Ab 6D4 (B) or MICA-specific Ab 2C10 (C) for 48 hours. The frequency of IFN- γ producing cells was determined by ELISPOT. * $P < 0.05$. This experiment was repeated twice with similar findings.

HSP70. This conclusion is based on the fact that a mAb against MICA alone completely blocked the activity of HSP70 (Figure 5C). It is possible that the level of MICB protein was not sufficiently high to trigger NKG2D activation. Further experiments to overexpress MICB, particularly in the setting when MICA is not expressed (40), shall resolve this possibility. Similarly, more studies need to be carried out to rule out a contribution by ULBP proteins (41).

The mechanisms by which HSP70 induces MICA expression on DCs are unclear. Promoter analysis has demonstrated the presence of heat shock elements in the promoter region of both *MICA* and *MICB* (42). Indeed, *MIC* expression was reported to be inducible by heat shock. In light of the fact that HSP70 can travel "across" the plasma membrane to enter the nucleus (43), it is conceivable therefore that HSP70 might facilitate induction of *MIC* transcription upon complexing with the transcription factor heat shock factor-1. Equally plausible, extracellular HSP70 might serve as a chaperone to stabilize the cell surface MICA molecule or the NKG2D-MICA complex to enhance NKG2D downstream signaling.

The importance of the NKG2D-MICA interaction in HSP70-mediated IFN- γ release also demonstrated convincingly that NK cells rather than DCs are the source of IFN- γ , since NKG2D is expressed by NK cells but not by DCs. This point was further corroborated by qPCR analysis which showed no IFN- γ mRNA in DCs that were stimulated with HSP70 (data not shown). Our finding does not dispute the possibility of DCs to produce IFN- γ in other conditions, which remains a contentious issue (44). The

significant but low number of IFN- γ producing cells (50-200 per 10^5 cells) in HSP70-stimulated NK-DC co-culture makes further intracellular staining and phenotypical analysis difficult. We did however look into the possible impact of HSP70 on other aspects of NK function. We found that there was no induction of the granzyme B level by a quantification assay (data not shown), indicating that HSP70 does not appear to impact on the lytic function of NK cells. This finding is not surprising since the generation of IFN- γ does not have to necessarily parallel the lytic function of NK cells (45). For example, it is well known that the CD56^{high} NK cell population is the main producer of IFN- γ , whereas CD56^{dim} NK cells are more lytic (46). It was also reported that a high ratio of NK cells to DCs can lead to killing of immature DCs by NK cells (47), which was not systematically addressed in our system. Further studies will tackle these questions, including which subsets of NK cells can be preferentially activated by HSP70-primed DCs.

In summary, we have unveiled that extracellular HSP70 could serve as an important positive regulator of DC-NK cell interaction, which should have implications in the understanding of the fundamental mechanisms of the immune response. Our findings should also be useful for improving HSP vaccine design and immunotherapy of cancers.

Abbreviations

HSP70, heat shock protein 70; MICA, MHC class I chain-related protein A; MoDCs, monocyte-derived DCs

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Materials and methods

Patients

Study subjects were either normal volunteers or patients diagnosed with chronic phase CML, as detailed previously (21). The clinical protocol was approved by the U.S. Food and Drug Administration, as well as the Institutional Review Board of the University of Connecticut Health Center. Leukocytes were obtained by leukapheresis. All CML patients received weekly HSP70 vaccine purified from autologous leukocytes, at 50 µg per dose intradermally, for a total of eight doses. Peripheral blood was obtained two weeks prior to the first vaccine (designated 'pre'), one week after the fourth vaccine (designated 'mid'), and two weeks after the eighth vaccine (designated 'post') for immunological analysis.

Preparation of HSP70

Leukocytes were washed with sterile phosphate buffered saline (PBS) and Dounce homogenized as described (48). In brief, HSP70 was purified using ADP-affinity chromatography,

followed sequentially by Blue-Sepharose column to remove serum albumin and by DEAE anion exchange chromatography. The final product was homogenous as demonstrated by a single 70 kDa band on both silver staining and Western blot with a monoclonal antibody (mAb) against HSP70 after separation on a 10% SDS-PAGE (21). These clinical grade HSP70 preparations, purified under good manufacturing practice (GMP) conditions, were uniformly free of endotoxin (<0.05 EU) as verified by a chromogenic limulus amoebocyte assay (data not shown).

Antibodies, cells and IFN- γ ELISPOT

Antibodies (Ab) against NKG2D (1D11) (26) and MICA (2C10) (49) were initially kindly supplied by Thomas Spies (Fred Hutchinson Cancer Center, Seattle) and were later purchased, along with other Abs, from BD Pharmingen (San Diego, CA). The KG1 cell line was kindly provided by Peter Cresswell (Yale University, New Haven, CT) (25). Monocyte-derived DCs (MoDCs) were derived from peripheral blood mononuclear cells (PBMCs) in the presence of GM-CSF and IL-4 without significant modifications (50). CD56+ NK cells were enriched from PBMCs by a magnetic separation protocol using magnetic beads conjugated with Ab against CD56 according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). The purity of the CD56+ cells at the end of each separation was consistently over 90% and verified by flow cytometry (data not shown).

The IFN- γ ELISPOT assay was previously described in detail (21). In this study, the effector cells employed were whole PBMCs, CD56+ NK cells co-cultured with DCs for 48 hours, in the presence or absence of HSP70, or a control protein bovine serum albumin (BSA) resuspended in the same buffer. In some experiments, control Ab or blocking Ab against NKG2D, pan-HLA class I (clone W6/32), MICA or MICB were added to the culture to determine the contribution by NKG2D-MIC interactions.

Flow cytometry

For the detection of MICA, MoDCs were treated with BSA or HSP70 at 20-100 μ g/ml for two days. After washing and blocking with staining buffer (PBS with 2% fetal bovine serum and 0.09% NaN₃), cells were pelleted and incubated with Ab against MICA, followed by staining with fluorochrome-labeled CD11c or secondary Abs at the appropriate dilution and incubated for 30 minutes at 4°C. After staining, cells were then washed and analyzed on flow cytometry using FlowJo software (TreeStar, Ashland, OR). Dead cells were always gated out by propidium iodine (PI) exclusion.

Statistical analysis

Student's *t* test was used to determine if the difference of an outcome as a result of a single intervention was statistically significant. *P* values <0.05 were considered to represent statistically significant differences.

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