Hsp70 induces Th1 polarization through tumor-associated macrophages in a T-cell lymphoma

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Tumor progression produces immunoregulatory phenotype of macrophages in tumor bearing host (TBH), that mediate immunosuppression through increased production of soluble factors. These factors obviously suppress the T-cell responsiveness and underproduction of Th1-polarizing cytokines. Here, we reported that *in vitro* treatment of TAMs with autologous Hsp70 purified from DL-bearing mice reverse back the tumor induced macrophage suppressor activity, suggesting that Hsp70 can restore TAMs production of Th1-polarizing cytokines. LPS stimulation failed to overcome tumor-induced dysregulation of IL-1, IL-12, IL-15 and IFN-γ production. In contrary, Hsp70 significantly enhanced IL12, IL-15, IL-1 and IFN-γ production by TAMs *in vitro* and *in vivo*, but also enhanced the LPS and IFN-γ responsiveness of TAMs. These Th1 polarizing effects of cytokines of TAMs are dose dependent and reach the maximal values at 24 hrs of incubation. Though, we found a significant release of IFN-γ in TAMs without T-cells, and increased level of IFN-γ with T-cells suggests that Hsp70 stimulates T-cells. Summarizing, these data demonstrates that Hsp70 restore Th1 polarizing cytokines production in the TBH and thus ascribe a possibility to develop a novel immunotherapeutic regime by using TAMs that could contribute well to the correction of tumor induced immune dysfunction.

Key words: Hsp70, IL-12, IL-15, IFN-γ, TAMs, Th1 Polarizing adjuvant.

Heat Shock Proteins (HSPs) are molecular chaperones constitutively expressed in all prokaryotes and eukaryotes. They are diverse class of intracellular protein molecules, whose expression is up regulated under stress conditions such as anoxia, infection and malignancies. HSPs act as a molecular chaperone by binding to unfolded or partially folded polypeptides, and prevents their misfolding, unfolding and aggregation of misfolded proteins, thereby protecting the cells from stress insults due to aggregation of misfolded proteins. HSPs are classified into several families on the basis of sequence homology. Among all HSPs, Hsp70 is most abundant [1] and may account for 2-5% of total intracellular proteins. HSP have been obtained increasing interest, since it has been shown that HSPs-like Hsp70 [2,3] and gp96 [4] purified from tumor cells are capable of enhancing the immunogenicity of tumors and eliciting protective cytotoxic T-lymphocytes (CTL) immune response even in the absence of $CD4^+$ T-cell, through antigen presentation directly by MHC class I molecules. However, it has been also reported that it also enhances the presentation of tumor antigen through MHC class II molecules [5] and producing strong immunological memory to protect the host from subsequent challenges of the same tumor. The increased immunogenicity of HSP is based on antigenic peptides associated with Hsp70 and gp96 molecules [6] through uptake of HSP by antigen presenting cells (APC) mediated by α-$_2$ macroglobulin receptor CD91 [7,8] expressed on APCs.

Autologous HSP preparations are delivering signals to the immune system irrespective of antigenic peptides associated with them, and activate a number of cells of innate immune system. It has been reported that Hsp70 either of microbial or human origin are able to activate macrophages and dendritic cells (DCs) in both murine as well as human cells [9] to produce non-specific effector molecules [10], pro-inflammatory cytokines, various chemokines and up regulation of co-stimulatory molecules in APCs. It was reported that Hsp70 signaling is stimulated by CD14 [11], which is a co receptor of Toll-like receptor-2 (TLR-2), originally recognized as the LPS receptor.

The macrophages are considered as main line of host defense and play vital role against malignancies including tumor

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cytotoxicity and stimulation of T-lymphocytes [12]. However, tumors circumvent these host-mediated immune activities through the production and release of immunosuppressive factors, such as IL-10, PGE_2 and TGF-β that adversely alter macrophage function and phenotype. These tumor-derived factors suppress the macrophages and inhibit T-cells responses making macrophages tumoricidally dysfunctional [13]. These tumor-associated macrophages (TAMs) are low producers of reactive nitrogen intermediate [14] and reactive oxygen intermediate [15]. In addition, they are found to be little responsive and even irresponsive to LPS [16] and IFN-γ [17], which are potent immunomodulatory agents [18]. These functionally skewed population of TAMs are polarized M_2 phenotype of macrophages manipulation of which may increase opportunities for immunotherapeutic intervention.

Although, there are ample evidences that HSPs such as Hsp60 [19] and Hsp70 [20] have potential to induce innate immune system by activating macrophages [21]. But all these works are oriented to the effect of preparations on normal macrophages and the various murine as well as human cell lines of monocytic lineages. The immunomodulatory function of Hsp70 on tumor-associated macrophages (TAMs) either *in vitro* or *in vivo* has not been studied. Therefore, the present investigation is intended to know the role of Hsp70 on the immunomodulatory function of tumor-associated macrophages (TAMs) *in vitro* and *in vivo*, and whether the treatment of Hsp70 reverse the tumor-induced suppressor activities through the reconstitution of macrophage IL-12 and IL-15 production in the TBH and enhanced production of Th1 polarizing cytokines. It is well documented that macrophages are most abundant at the site of tumor, and modulating the function of TAMs will be immunotherapeutically much relevant against malignancies.

Material and methods.

Mice and Tumor system. Pathogen free BALB/c ($H\text{-}2^d$) inbred adult mice of either sex at 8–10 weeks of age were used. The mice received food and water *ad libitum* and were treated with utmost human care in an approved animal room facility. For tumor system, Dalton's lymphoma (DL-cells), a type of T-cell lymphoma were maintained in ascitic form by serial transplantation in BALB/c mice, and stock of DL-cells is also maintained in a cryopreserved state for reference. In all the experiments, peritoneal macrophages and DL-cells as applicable were obtained, where the yield of cells is higher. To induce T-cell lymphoma, mice were injected intraperitoneally with 1.5 X 10^6 cell/mouse in 1ml phosphate buffer saline (PBS).

Reagents. Tissue culture medium RPMI-1640 from Hi Media (India) and fetal calf serum (FCS) was purchased from Hyclone (Logan, Utah), Lipopolysaccharide (*E. coli*) and most of other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo). Concanavalin-A (Con-A) and Goat IgG conjugated with alkaline phosphatase were obtained from Bangalore Genie (India). Murine recombinant IFN-γ was obtained from NIBSC (Hertfordshire, UK) and recombinant cytokines; IL-2 and IL-15 were obtained from Chemicon Co. Antibodies against IL-4, IL-10, IL-12 and IL-15 and IFN-γ were obtained from Imgenex (San Diego, CA).

Purification of Hsp70. The Hsp70 were isolated and purified as previously described [22] with minor modifications. A total 10ml of packed of DL-cells, cultured *in vitro* in thermal stress condition, was homogenized in 40 ml of hypertonic buffer A without detergent (10mM $NaHCO_3$, 0.5mM PMSF, pH 7.1) and centrifuged at 1,00,000 x g. The pellet was dissolved in buffer B (20mM NaCl, 15mM 2-ME, 3mM $MgCl_2$, 0.5mM PMSF, pH 7.5) using Sephadex column (G-75 from Sigma). The elute was loaded on ADP or ATP-agarose column (Sigma) equilibrated with buffer B. The column was washed extensively with buffer B until protein was undetectable in the elute by absorbance at 280nm. The buffer of the elute from ADP or ATP-agarose column was changed for buffer C (20mM Na_2PO_4, 20mM NaCl, pH 7.0) and the elute was loaded onto a DEAE-Sephacel column (Sigma) in buffer D (130mM NaCl in buffer A). The protein content in the different fractions was measured by the Lowry method with BSA as standard.

Assay for the endotoxin content. Hsp70 preparations and reagents used were determined for LPS content by Limulus amoebocyte lysate assay and found under a sensitivity limit of 0.1ng/ml.

Isolation and Activation of Macrophages. Macrophage monolayer was prepared from both normal and DL-bearing mice of either sex by a standard method. Both normal host (NH) and tumor bearing host (TBH) were killed by cervical dislocation and macrophages were harvested by peritoneal lavaging as peritoneal exudates cells (PEC) using chilled serum free culture medium RPMI-1640. For isolation of macrophages from TBH mice, PECs were harvested only after 7 days of DL-cell transplantation. PEC was cultured in round Petri dishes (Tarson, India) at 37^0C in CO_2 incubator (Sheldon, Oregon) for 2hrs. The culture were washed three times with warm serum free medium with gentle flushing to ensure that all DL and other non-adhering cells were removed. More than 95% of the adhering cell population was that of macrophages as determined by morphology and non-specific esterase staining. The PECs were detached from the petridishes using a cell scraper (Corning, USA) and seeded in 96-well flat bottom culture plates (Tarson, India) at a cell density of $1.0x10^5$ cells/well. The cells were then incubated in medium alone or medium containing different concentration of Hsp70, LPS, Hsp70+PmB or Hsp70+ antibody against Hsp70 for 24hrs or for different time intervals.

Isolation of T-cells. For T-cell isolation, thymus of TBH were removed and crushed onto a slide. The cells on slides are then washed by pipette with PBS and centrifuged at 2000x g for 5min. at 4^0C to remove unwanted materials and cell debris. The cell pellets were suspended in PBS and again centrifuged for 5min. The cell pellets were finally re suspended in RPMI-1640 medium supplemented with 10% FCS for 2hrs.

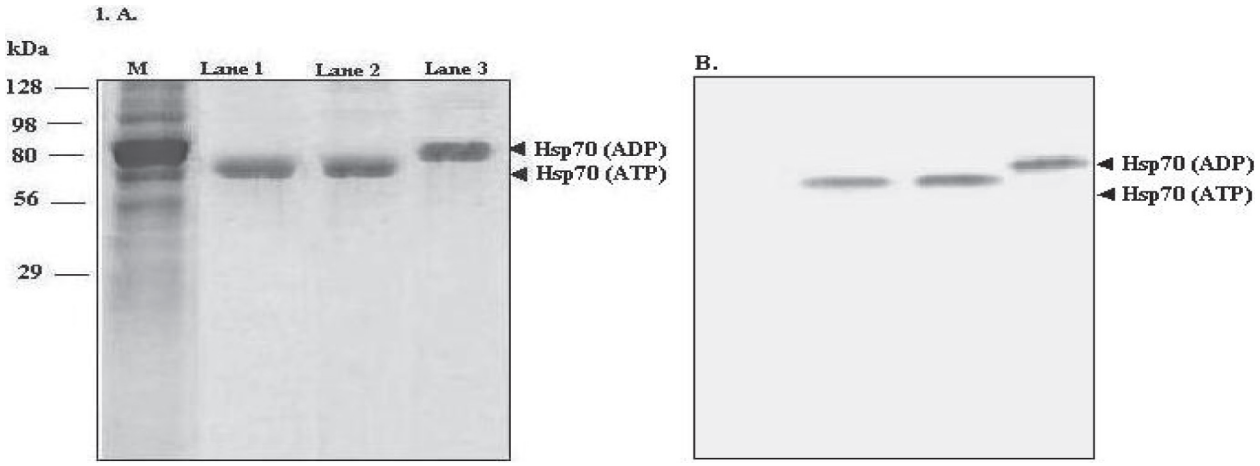

Fig.1. Characterization of Hsp70 fraction. Hsp70 isolated from ATP/ ADP agarose-column was characterized by SDS-PAGE with molecular weight markers. The bands obtained were transferred to nitrocellulose membrane for immunoblot with specific monoclonal Hsp70 antibody as described in materials and methods. Lane 1, band of Hsp70 standard; Lane 2, the Hsp70 eluted from ATP column; and Lane 3, the Hsp70 eluted from ADP column.

The non-adhering cells were removed by washing three times with serum free culture medium.

Cytokine assay. Values of cytokine released into culture supernatants were determined of double sandwich ELISA. Briefly, 96 well microtitre plate (Tarson, India) was coated overnight at 4 °C with 50µl of monoclonal antibodies against IL-1, IL-4, IL-10, IL-12, IL-15 and IFN-γ diluted with coating buffer (0.1mM $NaHOC_3$, pH 8.2). After discarding the coating buffer and two washes with washing buffer (PBS/ 0.05% Tween20, pH7.4), the wells were blocked with 200µl of blocking buffer (PBS/ 2% BSA) for 1hrs at 37 °C. The plates were then washed thee-times with washing buffer. Thereafter 50µl of standard for IL-1, IL-12, IL-15 and IFN-γ, and the same amount of culture supernatants was added in appropriate wells. The plates were then incubated for 2hrs at 37 °C. The plates were washed again three times and there after, 100µl of goat IgG conjugated with alkaline phosphatase with dilution of 1:5000 was added in each well and incubated for 1hr at 37 °C. The plates were then extensively washed and incubated with p-nitro phenyl phosphate (1mg/ml enzyme substrate buffer) for 45min. at 37 °C for color development. The absorbance was read at 450nm of wavelength and the concentration of cytokines in the culture supernatants were determined by calculating the absorbance of known concentration of standard. The reading of IL-4 and IL-10 was presented as absorbance due to unavailability of standard.

T-cell activation. Purified naive T-cells obtained from DL-bearing mice were seeded in 96-well flat bottom culture plate at the cell density of $2x10^4$ cells/well and incubated for 18 hrs in medium alone or medium containing 10µg of Hsp70 (ATP). In another set of experiment, the same cell density of T-cells were co-incubated with $1.0x10^5$ PEC pre activated with 10µg of Hsp70 (ADP) or Hsp70 (ATP). For IFN-γ quantification, supernatants from individual cultures were collected and analyzed. Incubating T-cells with medium alone or co-incubated with controls containing macrophages treated with medium alone.

MTT-assay. MTT-assay was carried out to estimate proliferation of T-lymphocytes upon stimulation. For the experiment $2x10^4$ T-lymphocytes were co-incubated with $1.0x10^5$ macrophages from NH or TBH previously treated with medium alone or medium containing LPS, Hsp70 (ADP) or Hsp70 (ATP) in 96-well round bottom culture plate. Thymocytes were obtained from mice of 9–10 weeks of age injected intra peritoneally $1.5x10^6$ DL-cells. Thereafter, MTT-assay was done as described [23]. Briefly, MTT [3-(4,5-dimethythiazol 2-yl)-2,5-diphenyltetrazolium bromide] was dissolved in PBS at a concentration of 5.0 mg/ml. 50ml of MTT solution was added to each well of culture plate and incubated for 37 °C for 4hrs. The plate was centrifuged for 5min at 100x g at 4 °C.Supernatent was then carefully removed without disturbing the dark-blue formazon crystals, and 100µl of DMSO was added to each well to dissolve the formazon crystals and the reading was taken by UV/VIS spectrophotometer at 540 nm of wavelength.

Immunization. For immunization, TBH as well as NH mice were used. Autologous Hsp70 from either fraction were injected intra-peritoneally in a dose of 50µg. After 4 days, 50µg Hsp70 was again injected as a booster dose and thereafter-peritoneal fluid and ascitic fluid were taken by 22-gauze syringe from the peritoneum of NH and TBH mice respectively for analysis. Mice injected with PBS were taken as control group.

SDS-PAGE and Immunoblot. Samples of purified Hsp70 were denatured by boiling for 5min in SDS sample buffer and resolved in 15% SDS-PAGE together with recombinant Hsp70. After electrophoresis, proteins were stained in

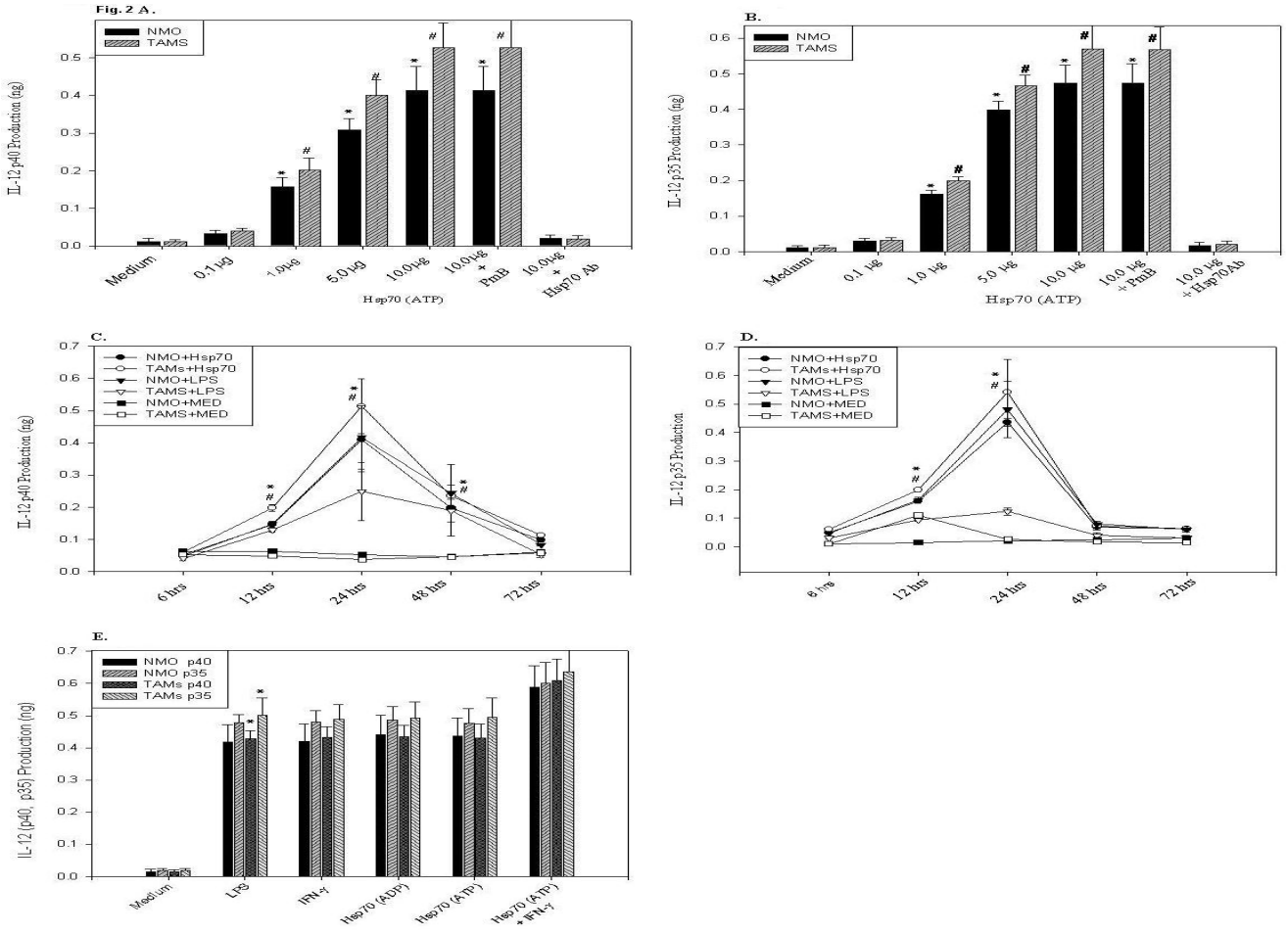

Fig.2. IL-12 Production. 1.0x10^5 NMO and TAMs harvested either from mice before immunization or after immunization were incubated with medium alone or medium containing indicated concentrations of Hsp70 (ATP), 10.0 µg of Hsp70 (ATP) + Polymyxin B (PmB) or 10.0 µg of Hsp70 (ATP) + Hsp70 Ab for 24 hours, or incubated with medium alone or medium containing 10 ng LPS, or 10.0 µg of Hsp70 (ATP) for different time intervals, and IL-12 p40 and p35 were measured in culture supernatant. Fig A, shows dose dependent IL-12 p40 production; B, dose dependent IL-12 p35 production; C, time dependent IL-12 p40 production; D, time dependent IL-12 p35 production; and E, IL-12 p40 and p35 production by NMO and TAMs harvested from immunized mice. Data represent the mean concentrations ± SEM of three independent experiments in triplicate. The symbols * and # indicate that data is significant at p<0.05.

Coomasie blue stain then transferred to nitrocellulose membrane (Sartorius, Germany) and probed with rat anti-Hsp70 polyclonal antibody (Chemicon) at 1:2000 dilutions. Goat-alkaline phosphatase conjugated antibodies (Bangalore, Genie, India) was used as a secondary antibody at a dilution of 1:7500. Finally, the blots were incubated with 5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium reagent for 5-10min. Equal protein loading was verified by re probing the blots with anti-actin Ab (Santa Cruz Biotechnology).

Statistical Analysis. All experiments conducted in triplicate at least for three times. Simple two-tailed Student t- test and ANOVA were done, and significant level was assessed at p is less than 0.05. The Sigma plot Version 5.0 was used for statistical analysis of the data.

Results

Hsp70 analysis. Hsp70 were isolated and purified from DL-cells in ascitic form harvested from DL-bearing mice 7 days after DL-cells transplantation as described in materials and methods. The Hsp70 purity was estimated to be >95% as determined by the staining of the SDS-PAGE by Coomasie blue stain of Hsp70 standard (Fig.1.a). The bands were reconfirmed by immunoblot using Hsp70 antibodies recognizing both Hsp70 and Hsc70 (Fig.1.b).

Induction of IL-12 production in TAMs by Hsp70. Production of IL-12 was assayed in the culture supernatants of PEC harvested from both NH and TBH mice, following *in vitro* treatment with Hsp70 preparations (both Hsp70 ATP and

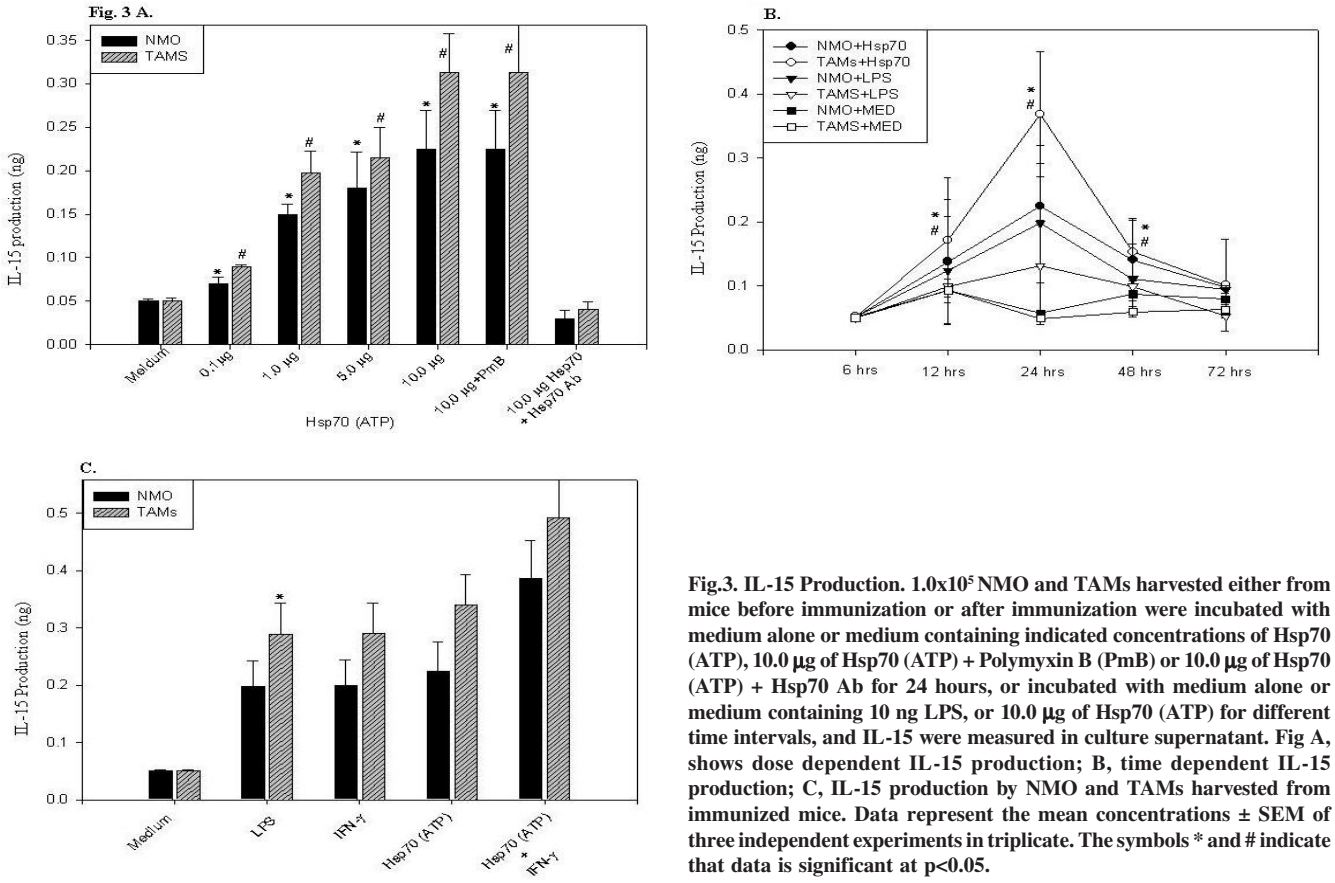

Fig.3. IL-15 Production. 1.0x10^5 NMO and TAMs harvested either from mice before immunization or after immunization were incubated with medium alone or medium containing indicated concentrations of Hsp70 (ATP), 10.0 µg of Hsp70 (ATP) + Polymyxin B (PmB) or 10.0 µg of Hsp70 (ATP) + Hsp70 Ab for 24 hours, or incubated with medium alone or medium containing 10 ng LPS, or 10.0 µg of Hsp70 (ATP) for different time intervals, and IL-15 were measured in culture supernatant. Fig A, shows dose dependent IL-15 production; B, time dependent IL-15 production; C, IL-15 production by NMO and TAMs harvested from immunized mice. Data represent the mean concentrations ± SEM of three independent experiments in triplicate. The symbols * and # indicate that data is significant at p<0.05.

Hsp70 ADP). Since, the bioactive IL-12 heterodimer is depending upon the constitutively expressed p35 subunit and the inducible p40 subunit. We analysed the effect of Hsp70 preparation on the expression of both the subunits separately.

We observed that NMO and TAMs cultured in complete RPMI-1640 medium with or without 0.1 µg of Hsp70 produced little IL-12 p40 (L 0.035 ng/ml) and p35 (L 0.0313 ng/ml) and thereafter, stimulation with increasing concentrations of either Hsp70 (ATP) or Hsp70 (ADP), a dose dependent increase in the concentration of either subunit was observed (data of Hsp70 ADP are not shown due to almost similar effect as that of Hsp70 ATP). A significant amount of both subunits in the culture supernatant of NMO and TAMs were observed only after treatment with 1.0 µg of Hsp70, and level reached to maximum following the treatment with 10.0 µg of Hsp70 (Fig.2.A & B). To exclude the possibility of effect due to endotoxin content, we treated the NMO and TAMs with 10.0 µg Hsp70 with 10.0 µg of PmB and did not find any abrogation in the production of either p40 or p35, while on addition of Hsp70 mAb completely abrogated the effect. In each condition, we found a significant difference in the production of p40 and p35 subunit in NMO and TAMs. When NMO and TAMs harvested from immunized mice were stimulated with Hsp70 *in vivo*, showed almost similar response, but here, we found that following immunization, TAMs become more responsive to LPS than NMO (Fig. 2C).

Both the subunits of IL-12 were detected significantly as early as 12 hrs of stimulation and their production was maximal at 24 hrs in incubation (Fig. 2 D& E). After that, sharp decrease in production was observed in culture supernatants of both NMO and TAMs. Here, we found that NMO is more responsive to LPS in comparison to TAMs, but for LPS too, they follow similar dynamics.

Effect on IL-15 production. To identify, whether Hsp70 would induce the production of IL-15 in TAMs; we incubated NMO and TAMs with medium alone or with medium containing different concentration of Hsp70 (only Hsp70 ATP). We observed that the minimal level of Hsp70 to induce IL-15 production is 1.0 µg in this case also, whereas, treatment with 0.1µg Hsp70 showed insignificant level of IL-15 in the culture supernatants of both NMO and TAMs. However, in any case, TAMs showed higher level of IL-15 production in comparison to NMO (Fig.3 A) and maximal production was found at 10.0 µg of Hsp70. Before immunization, *in vitro* treatment

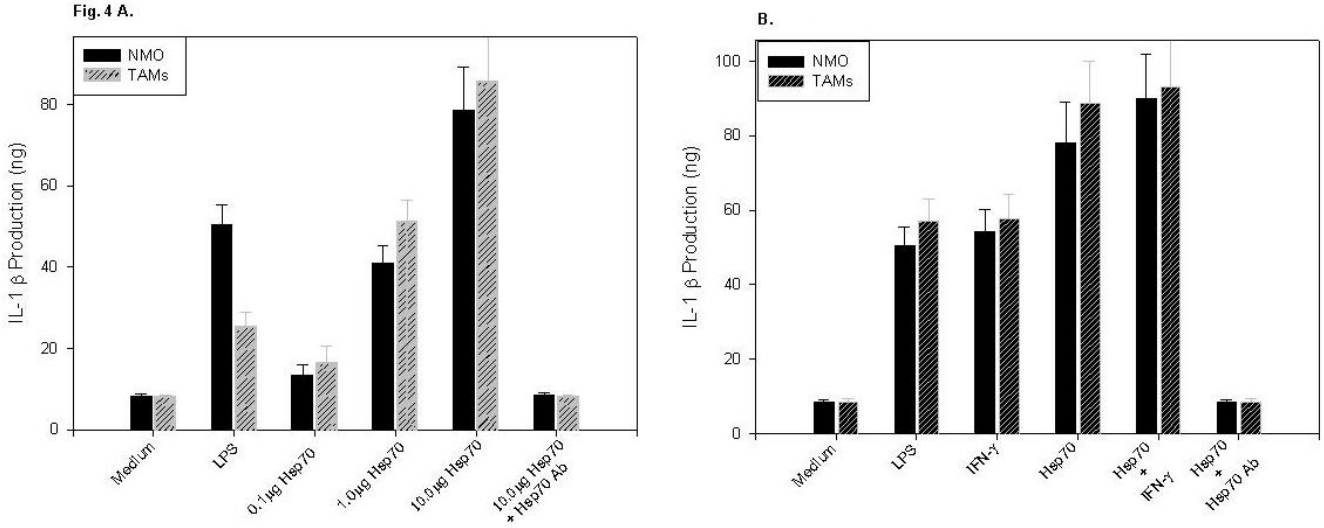

Fig. 4. IL-1β Production. 1.0x10^5 NMO and TAMs were incubated with medium alone or medium containing 10 ng LPS, or 10.0 µg of Hsp70 (ATP) or 10.0 µg of Hsp70 (ADP) for 24 hours, and absorbance at 540 nm was read for IL-1ß production in culture supernatants. The data represent the mean absorbance ± SEM of three independent experiments in triplicate. The symbols * and # indicate that data is significant at p<0.05

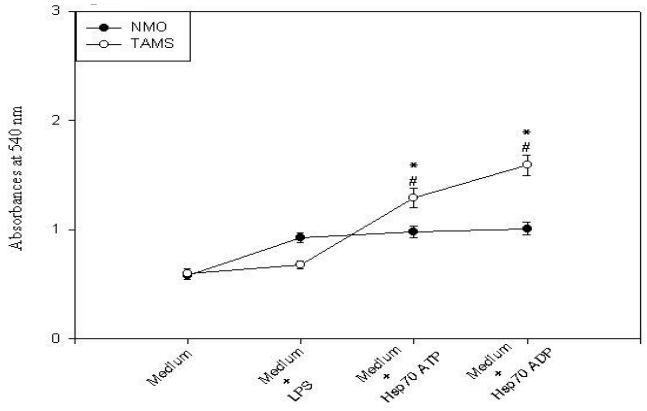

Fig. 5. Thymocyte Proliferation. 1.0x10^5 NMO and TAMs were incubated with medium alone or medium containing 10 ng LPS, or 10.0 µg of Hsp70 (ATP) or 10.0 µg of Hsp70 (ADP) for 24 hours and 2x10^4 thymocytes were added in each well, and MTT assay was done as described in material and methods. The absorbance was measured at 540 nm and data are represented as the mean absorbance ± SEM of three independent experiments in triplicate. The symbols * and # indicate that data are significant at p<0.05.

of LPS, TAMs were found to be low responder (data not shown), but TAMs recovered from immunized mice showed a marked increase in responsiveness against LPS, with maximum at 24 hrs (Fig. 3 B).

An IL-15 in culture supernatants of both populations of macrophages was detectable in significant amount not earlier than 12 hrs of incubation and maximum production was found after incubation of 24 hrs in time kinetics of *in vitro* stimulation. Thereafter, a marked decrease in the level of IL-15 was observed, and after 72 hrs of incubation, it almost reached minimum (Fig. 3 C).

Production of IL-1β. IL-1β production was measured in the culture supernatants of peritoneal exudate cells harvested from both NH and TBH mice following *in vitro* treatment with different concentration of Hsp70 (ADP) preparation. Maximal production of IL-1β was observed on 10.0µg of Hsp70 whereas, 0.1µg of Hsp70 have no any effect in both cases. Heat treated Hsp70 (data not shown) and 10.0µg of Hsp70 with Hsp70 antibody completely abrogate the effects. The PECs harvested from TBH mice showed lower production of IL-1β than the PECs harvested from NH (Fig. 4 A), but after immunization, PEC recovered from TBH mice showed significantly higher production of IL-1β following LPS treatment (Fig. 4 B) than the PECs recovered from NH mice indicating that Hsp70 administration increases the LPS responsiveness of PECs. The IL-1β production was found to be enhanced following the treatment of IFN-γ in PECs of post immunized mice.

*Effect of Hsp70 on T-cell Proliferation***.** We, further, investigated whether, Hsp70 has any role in T-cell proliferation? We have done MTT-assay for thymocyte proliferation as described in materials and methods using both fractions of Hsp70 isolated from either ATP-agarose column or ADP-agarose column. The culture supernatants of NMO and TAMs was incubated in medium alone had no effect on T-cell proliferation, and supernatants of LPS treated NMO and TAMs showed significant increase in absorbance at 540 nm (Fig 5), and LPS treated NMO were used as controls. However, supernatants of TAMs previously treated with LPS showed only a marginal

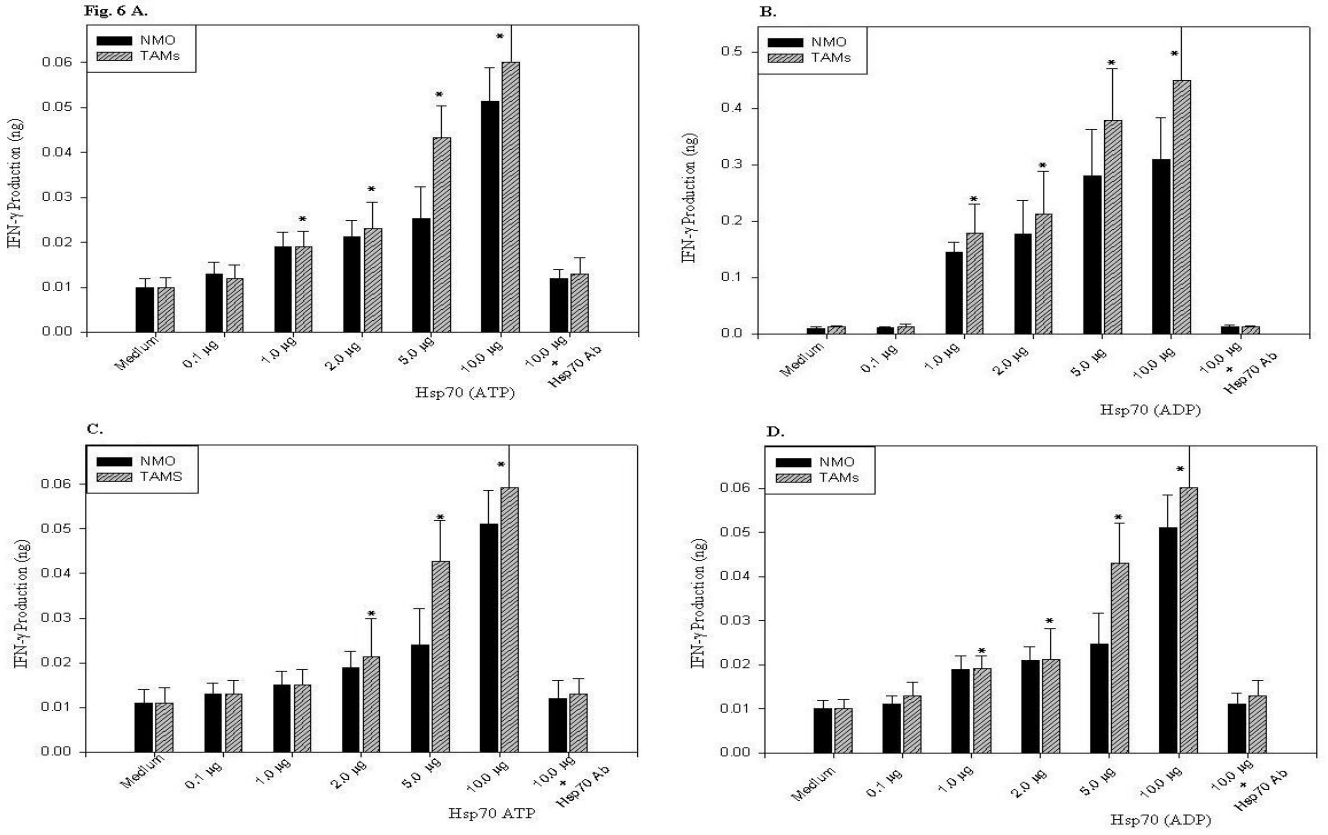

Fig.6. IFN-γ Production. 1.0x10^5 NMO and TAMs were incubated with medium alone or medium containing indicated concentrations of Hsp70 (ATP) or 10.0 µg of Hsp70 (ATP) + Hsp70 Ab/or Hsp70 (ADP), Hsp70 (ADP) + Hsp70 antibody for 24 hours with and without T-cells and the IFN-γ level was measured in culture supernatant. Fig A, shows IFN-γ production by NMO and TAMs incubated with Hsp70 (ATP) fractions without T-cells; B, IFN-γ production by NMO and TAMs incubated with Hsp70 (ADP) fractions without T-cells; C, IFN-γ production by NMO and TAMs incubated with Hsp70 (ATP) fractions and with T-cells; D, IFN-γ production by NMO and TAMs incubated with Hsp70 (ADP) fractions and with T-cells. Data represent the mean IFN-γ concentration ± SEM of three independent experiments in triplicate.
The symbol * indicates IFN-γ levels from TAMs treated with Hsp70 (ATP) than those from NMO treated with Hsp70 (ATP) at p<0.05.

increase in T-cell proliferation compared to NMO treated with LPS. But, when T-cells were incubated with culture supernatant of TAMs that was previously incubated with medium alone or medium containing Hsp70 ATP showed significant and much higher proliferation (absorbance 0.149 ± 0.09 nm) in comparison to the culture supernatants of NMO incubated with Hsp70 ATP (absorbance 0.98 ± 0.055 nm). Hsp70 ADP treatment showed only marginal increase in T-cell proliferation compared with Hsp70 ATP.

Hsp70 induces IFN-γ Production. To determine, whether the Hsp70 have any augmentary effect on IFN-γ production by macrophages and activation of DL-specific T-cells, we analysed the culture supernatants of macrophages incubated with medium alone or medium containing different concentration of Hsp70 (ATP), Hsp70 (ADP) with (Fig. 6 A & B) or without (Fig. 6 C & D) the addition of DL-specific T-cells. We observed that similar to IL-12 and IL-15 production, NMO and TAMs responded well for IFN-γ production, and IFN-γ in culture supernatant of NMO and TAMs was found maximal with 10.0 µg of Hsp70 treatment. The treatment with Hsp70 specific antibody completely abrogated the effect of Hsp70. On addition of T-cells with NMO and TAMs, higher level (>0.05 ng) of IFN-γ in culture supernatants was observed (0.145 ± 0.018 ng for NMO and 0.179 ± 0.052 ng of TAMs) when treated with Hsp70 ADP. Significant differences in the level of IFN-γ were observed in supernatants of T-cells incubated with NMO and TAMs previously treated with Hsp70 ATP. The maximum production of IFN-γ was observed on treatment with 5.0 µg of Hsp70 ADP, and later only a marginal increase in IFN-γ production was observed in culture supernatants.

*In vivo treatment decreases Th2 cytokines production***.** Interestingly, it was found that before immunization of TBH mice, the level of Th2 polarizing cytokines IL-4 and IL-10 in

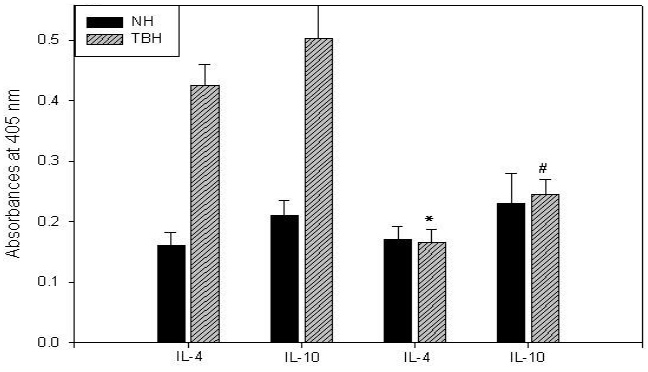

Fig.7. Th2 Cytokine Level. Ascitic fluid of tumor-bearing host (TBH) mice was collected before and after immunization of 50 μg of Hsp70 (ATP) and level of IL-4 and IL-10 were determined at 405 nm absorbance. Data indicate mean absorbance ± SEM of three independent experiments in triplicate. The symbols * and # indicate p<0.05.

ascitic fluid (peritoneal fluid containing DL-cells in ascitic from) were found to contain a much higher concentration of IL-4 (0.90 ± 0.045) and IL-10 (1.11 ± 0.095) (Fig. 7). Before and after immunization of NH mice, the level was found unchanged, but after immunization of TBH mice, we found a sharp decrease in the level (0.165 ± 0.044 absorbance for IL-4 and 0.245 ± 0.055 for IL-10). This indicates that tumor causes the significant release of such Th2 dominating cytokines that was suppressed by immunization of TBH mice with either form of Hsp70.

Discussion

In addition to the well-established role of HSPs in eliciting strong and specific CTL response or adaptive immune response, evidences have emerged that HSP also stimulates innate immune system as well. It has been reported that HSP can activate macrophages and dendritic cells to produce non-specific effector molecules, pro-inflammatory cytokines, chemokines [24] and results in up-regulation of co-stimulatory molecules in dendritic cells [25–27]. The present study on the potential of HSPs to activate normal macrophages is consistent with previous work [32–35], but no work has been done on macrophage derived from tumor bearing host (TBH) either *in vivo* or *in vitro*; though it is the population of immune cells most abundant at the tumor sites. Tumor growth significantly alters phenotype and function of normal resident macrophages by releasing various immunosuppressive factors, such as IL-10, PGE2 and TGF-β. These tumor-associated macrophages (TAMs) get the tumor promoting characters in immunosuppressive micro-environment. Therefore, our recent studies on the immunomodulatory activities of Hsp70 is basically focused on the effects of Hsp70 on the function of TAMs, also designated as alternatively activated macrophages or polarized M_2 phenotype.

Our result shows that autologous Hsp70 preparations are able to activate TAMs in a manner similar to normal resident macrophages and enhances the production of IL-12 p40. The constitutively expressed p35 production is also enhanced following the treatment of Hsp70 both *in vivo* and *in vitro.* Likewise, the enhanced production of IL-15 by TAMs indicated that Hsp70 could revert back the normal functioning of TAMs in tumor micro-environment of TBH. Further, we observed that following exogenous application of Hsp70 in TBH, the LPS and IFN-γ responsiveness increases and after *in vitro* stimulation with LPS and IFN-γ, much higher production of IL-12 (p40 and p95) and IL-15 by TAMs was observed. The treatment of TAMs with heat-treated Hsp70 or Hsp70 with its antibody completely abrogated the production of these cytokines excluded the involvement of other such immune modifiers in the Hsp70 preparations. In addition to the enhanced production of IL-12 and IL-15, we found significant decrease in the level of IL-4 and IL-10 in ascitic fluid of tumor-bearing host following administration of Hsp70. Il-4 and IL-10 are Th2 cytokines that up regulate humoral immunity. They suppress the proliferation of Th1 cells and the production of Th1 cytokines viz., IL-12, IL-15 and IFN-γ. Therefore, the results indicate that Hsp70 treatment restores the Th1/Th2 imbalances and results into Th1 polarization in TBH through the restoration of suppressed function of TAMs. However, the production of these cytokines *in vivo* by dendritic cells can not be excluded. Though, after immunization, we found a significant but lower level of IL-4 and IL-10 in ascitic fluid of TBH that might also be produced by DL-cells.

Recognizing that tumor growth negatively regulates macrophage function in production of immunomodulatory soluble factors, it followed those therapeutic agents that reconstitute immune activity through indirect action could be inducing the production of stimulatory cytokines. Pretreatment or immunization of TBH with Hsp70 reverses suppressor activities of TAMs. This suggested that Hsp70, which have macrophage activating function *in vitro* and *in vivo*, might enhance T-cell reactivity by activating IL-12 and IL-15 production. HSPs are intracellular proteins and their expression is greatly up regulated in malignant cells and thereby increasing the local concentration much higher upon cell lysis. Therefore, in this consequence, one could speculate that the release of Hsp70 from necrotic tumor cells subsequently changes the antigen presenting function of TAMs *in situ*, in TBH, which in turn are able to induce clonal selection and expansion of naïve T-cells and their effector function. Our data suggest that Hsp70 treated TAMs produce IL-1 in adequate quantity, which is sufficient to induce T-cell proliferation. It is interesting to note that the treatment of TAMs with LPS comparatively could not achieve such parameters as well. However, LPS treatment of NMO produces much higher amount of IL-1. We observed a difference in IL-1 production on treatment with Hsp70 (ATP) and Hsp70 (ADP) (data not shown). This differences might be possible that Hsp70 (ADP) could deliver "invisible" antigens from intracellular antigenic pool of DL-cells to the

antigen presenting cells e.g. macrophages and by enhancing antigen presentation ability of macrophages in TBH, that activate T-cell to produce IFN-γ which further acts on TAMs to produce higher IL-1. Furthermore, we observed a pronounced level of IFN-γ production by T-cells co-incubated with TAMs, which were previously treated *in vitro* with Hsp70 (ADP). Though, Hsp70 is also able to induce the production of IFN-γ directly by NMO and TAMs, but the IFN-γ level is low, which shows that either exogenous application of Hsp70 or released after necrotic cell death, produces an efficient Th1 polarizing micro-environment that might be finally led to the induction of profound anti-tumor immune response.

The IL-12 and IL-15 are the potent Th1 polarizing cytokines, their production in TBH after immunization has important implication in using Hsp70 ATP/ ADP as a Th1 polarizing adjuvant. The IFN-γ production upon activation with Hsp70 treated TAMs culture supernatants indicate clearly that Hsp70 play a dual role in the course of T-cell activation and enhances the production of Th1 polarizing cytokines in TAMs and further enhances the activation of cytotoxic T-lymphocytes through these alternatively activated macrophages. Thus, present study expands our knowledge to understand the tumor-host relationship as well as TAMs reactivity with tumor cells, and also indicates that the immunosuppressive or tumor promoting function of TAMs could be reverts back towards tumor protection. Keeping the view of above observation, we will be able to develop a novel immunotherapeutic regimen by targeting tumor-associated macrophages. This study will be more beneficial and helpful for further preparation of HSP based tumor vaccines.

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