ANTIGENIC HSP70-PEPTIDE COMPLEX UPREGULATE ALTERED EXPRESSION OF DOCKING RECEPTOR ICAM-1 IN TAMS INCREASES IN DALTON'S LYMPHOMA BEARING MICE

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ABSTRACT

ICAM-1 facilitates docking between APC and T cell for cell-cell interaction and transmigration. Tumor progression downregulate the expression of ICAM-1 from M1 phenotype of macrophages into M2 phenotype macrophages which promote tumor progression. It has been observed that Hsp70 preparations derived from normal tissues do not elicit tumor immunity, while Hsp70 preparation from tumor cell able to elicit tumor immunity due to tumor antigen association. In the present study it has been investigated the function of Hsp70-peptide complex on TAMs in DL bearing host. For tumor system, healthy BALB/c (H2d) strain of mice of either sex at 8–12 weeks of age was taken and 1.0 X 106 DL cells in 0.5 ml sterile PBS were transplanted and obtained from ascitic fluid of DL-bearing mice. TAMs were harvested from tumor bearing mice as peritoneal exudates cells (PECs) by adherent purification. Hsp70 peptide complex was purified by peng et. al., 1998 method and hsp70 were used for treatment of cells. TAMs were treated with 10µg/ml of Hsp70 peptide for 24 hrs and expression profiling of ICAM-1receptors were analysis by Immunocytochemistry, western blotting, Flowcytometry and RT-PCR. Differences between groups were detected by unpaired t-tests. It has been observed that in normal state of M1 phenotype of macrophage the ICAM-1 expression was found (34.1±3.04) while in TAMs it was (9.0±1.2). Tumor progression decreased the expression of docking receptor CD54 or ICAM-1 over M1 phenotype which facilitates tumor progression by deactivation of cell mediated immune responses. Treatment of hsp70 peptide resulted in significant increase ICAM-1 expression in both normal phenotypes of macrophages (169±13.01) as well as TAMs (30.1±3.1) but it was at lesser extent as compare to macrophage.Therefore, it can be concluded that exogenous application of tumor derived hsp70 peptide enhances the suppressed ICAM-1 expression in Mϕ and TAMs, which provide further insight for DL-induced immunosuppression in a tumor-bearing host. Hsp70 peptide might be helpful in designing a novel immunotherapeutic vaccination approaches.

Key words: *ICAM-1; Macrophages; TAMs; Tumor Progression; Hsp70-peptide complex; Immunotherapy.*

INTRODUCTION

Intercellular adhesion molecule-1(ICAM-1or CD54) expressed (Huang *et al.,* 1995) on antigen presenting cells (APC) including macrophages belongs to Integrins family of glycoprotein. Integrins are ubiquitous /heterodimeric adhesion receptors that mediate cell to cell and cell to extracellular-matrix (ECM) interactions (Valentin *et al.*, 2006). The subfamily of integrins comprises a monophyletic group of closely related glycoprotein's critically involved in leukocyte adhesion and migration during the inflammatory immune responses (Kawakami *et al.,*2002) [3]. To the generation of effective macrophage/lymphocyte interaction requires direct cell to cell contact and docking between B cells and T cells. T cell activation is thought to require two distinct signals. The first signal (recognition) requires interaction of the TCR and MHC associated peptide complex and the second signal is provided by the CSMs on the antigen presenting cell and CD28 and CTLA-4 on the reacting T cell. If a costimulatory signal is not received, the T cell undergoes apoptosis instead of activation. Adhesion molecules, such as ICAM-1 act as a docking and receptor and further promote immune cell recognition and T cell stimulation upon interaction of other receptors such as TCR, B7 families and MHCs etc. Adhesion dependent leukocyte functions and immune responses such as CTL recognition and activation, B cell differentiation and inflammatory responses(Alison *et al.,* 2000).

Macrophages (Mϕ) are a fundamental cell of the innate defense system, which can promote specific immunity by inducing T cell recruitment and activation. Despite this, their presence within the tumor microenvironment has been associated with enhanced tumor progression and shown to promote tumor cell growth and spread, angiogenesis and immunosuppression by production of potent pro angiogenic cytokines (Antonio *et al.,* 2008) and growth factors, such as VEGF, TNF-α, TNF-β, IL-1, IL-6, IL-8 and bFGF that stimulate endothelial cell proliferation and promote the formation of differentiated capillary tube (Lin *et al.,* 2007) [6]. Additionally, they also express a broad range of angiogenic-modulating factors such as MMP-2, MMP-7, MMP-9, MMP-12 and cyclooxygenase-2 that play a significant role in capillary formation and vascularization in the tumor tissues (Chouaib *et al.,* 1997). These multifunctional MMPs have many substrates in the extracellular matrix and basement membrane, and are known to stimulate endothelial cell proliferation and migration, which can maintain tumor angiogenesis. This paradoxical role of macrophages in cancer finds an explanation in their functional plasticity that might result in the polarized expression of either pro or anti tumoral functions (Chouaib *et al.,* 1997). Key players in the setting of their phenotype are the microenvironmental signals to which macrophages are exposed, which selectively adjust their functions within a functional spectrum encompassing the M1 and M2 extremes.

HSPs are conserved group of proteins expressed in almost all type of cells. There are different types of HSPs and among all Hsp70 is highly conserved and immunogenic, capable of inducing both B and T cell activation. Hsp70 plays an important role in antigen presentation play important role in MHC class I cross presentation of tumor antigen (Jattela *et al.,* 1999). It can activate both normal peritoneal resident macrophages and tumor associated macrophages leading

to enhanced production of NO, H_2O_2, inflammatory cytokines, chemokines and in addition to its profound role in eliciting cancer-specific immunity against the tumor by virtue of their ability to bind tumor-specific peptides. It has been observed that Hsp70 preparations derived from normal tissues do not elicit tumor immunity, but it can activate macrophages (Blachere *et al.*, 1997; Tamura *et al.*, 1997; Udono *et al.*, 1993; Tokuda et al., 1993).

Most of the work has been reported that ICAM-1 is in involved in the pathogenesis of malignancies such as lymphoma, leukemia, melanoma, lung cancer, and multiple myeloma and their expression varies during infection and malignancies but the effect of Hsp70-peptide complex on M2 phenotype of macrophages was not investigated. Therefore, in the present study, we sought to investigate the effect of Hsp70-peptide isolated and purified from DL bearing mice on expression of adhesion receptor in both M1 and M2 phenotype of macrophages.

MATERIAL AND METHOD

Reagents

RPMI 1640 culture medium was obtained from HiMedia, Mumbai, India. Fetal bovine serum (FBS) was obtained from Invitrogen, CA, USA and Goat serum was prepared and heat inactivated in the lab for plastic petriplate coating to purify TAMs; Anti-Hsp70 antibody was obtained from Biolegand, San Diego, CA, USA; secondary antibodies from Bangalore Genei (India); CD54 (ICAM-1) conjugated with FITC and CD14 conjugated with PE from ebiosciences, San Diego, CA, USA; Goat IgG conjugated with alkaline phosphatase was obtained from Bangalore Genie, Banglore, India. Trizol were purchased, from Biobasic, USA; dNTPs were purchased, from Finnzyme, Biolabs, New England; Taq polymerase were purchased, from Genai, Banglore, India; The ATP/ADP agarose, G-75 sephadex column, phorbol 12-myristate 13-acetate **(**PMA), hoechst 33258 were obtained from Sigma Company, Banglore, India. Na_2HPO_4, KH_2PO_4, formaldehyde and trypsin were purchased from Qualigens, Mumbai, India, acetone form Rankem Ltd, Mumbai, India and glutraldehyde was obtained from Serva Electrophoresis, Heidelberg, Germany. All other chemicals otherwise stated were obtained from Qualigens, Mumbai, India.

Animals and tumor model

Inbred populations of BALB/c (H^{2d}) strain of mice of either sex were used at 8-12 weeks of age. All animals were kept in conventional cages (6 animals in each cage) and received unsterilized food and water *ad libitum*. Experimental animals were inspected daily for survival. All animals were kept and maintained in utmost care under the guidelines of Animal Ethical Committee.

For tumor system, healthy mice of either sex at 8 to 12 weeks of age were injected intraperitoneally (*i.p.*) with 1.0×10^6 DL cells in 0.5 ml sterile PBS. The DL cells for transplantation were obtained from ascitic fluid of DL-bearing mice, where the yield of the cells is higher and maintained in ascitic form *in vivo* by serial transplantation.

Isolation and purification of Hsp70

The Hsp70 were isolated and purified as previously described (Laemmli *et al.*, 1970; Tamura *et al.*, 1999) 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 × 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). The whole elute was loaded on ADP or ATP-agarose column equilibrated with buffer B. The column was washed extensively with buffer B until protein was undetectable in elutes by absorbance at 280nm. The buffer of elutes from ADP or ATP-agarose column was changed for buffer C. (20mM Na_2PO_4, 20mM NaCl, pH 7.0) and elutes were loaded onto DEAE Sephacel column in buffer D (130mM NaCl in buffer A). The supernatant was collected and protein concentration was determined by Bradford method (Bradford *et al.*, 1976). Protein was mixed with 6x loading dye and subjected to 12% SDS–PAGE, followed by wet electroblotting onto PVDF membrane. The membrane was blocked in 5% (w/v) fat free skimmed milk, incubated overnight at 4°C with anti-Hsp70 antibody (1:1000), washed in 1x PBS, incubated for 2 h at room temperature with ALP- conjugated goat anti-mouse IgG (1:1000), Finally, blots were incubated with nitro blue tetrazolium (NBT) reagent for 10 min at 37°C for proper visualization of desired bands. For loading control, the same membrane was probed for β-actin with alkaline phosphatase conjugated antibody (1:1000).

Purification and activation of Macrophages

Macrophages were harvested from normal healthy and tumor-bearing mice by standard method (Tameike *et al.*, 1996; Kunisch *et al.*, 2012). Briefly, mice were killed by cervical dislocation and macrophages were harvested by peritoneal lavage as peritoneal exudates cells (PECs) using chilled serum free culture medium RPMI-1640. PECs were cultured in Petri dishes (Tarson, Kolkata, India) at 37°C in CO_2 incubator (Shelab, Oregon, CA, USA) for 2 h. The culture was washed three times with luke warm serum free medium with gentle flushing to ensure that all DL and other non-adherent cells were removed and adherent cells were collected. Adherence purified macrophages were seeded in flat-bottom culture plates (Tarson, Kolkata, India) at a cell density of 1.5×10^6 per well in the culture medium with or without PMA (10 µg/ml) or Hsp70 (10 µg/ml) and incubated for time periods of 24 h.

Identification and characterization of macropahges

Adherent cells were washed in phosphate-buffered saline (PBS) and fixed for 15 min using 0.2% glutaraldehyde in PBS. The cells were stained for 1 h at pH 6.3 at room temperature with α-naphthyl butyrate as the substrate, with the addition of 36 mM NaF to control for the diffuse cytoplasmic staining that would. not be attributable to monocytes (Broderick *et al.*, 2006). The reaction was stopped by removing the solution of α -naphthyl butyrate-NaF and rinsing the cells with water. Cells were counted by light microscopy (magnification X400). Those cells that exhibited bright red, diffuse cytoplasmic staining were considered to be positive for nonspecific esterase. Adherent cells were also fixed in 95% ethanol, stained with Giemsa and examined with

a phase-contrast microscope. Further, macrophage purification was confirmed by flowcytometry analysis by using $CD14^+$ antibody conjugated with PE.

Cell viability

Peritoneal exudates cells (PECs) were harvested by peritoneal lavage using chilled serum-free culture medium RPMI 1640. The PECs were then transferred(MacLauchlan *et al.*, 2009; Broderick *et al.*, 2006) into a vented plastic tissue culture flask (Tarson, Kolkata, India) for culture at 37°C in CO_2 incubator (Shella, Oregon, CA, USA). The non-adherent cells were discarded by washing three times with lukewarm serum-free culture medium with gentle flushing. After incubation, control group viability of peritoneal macrophages was determined using exclusion by the trypan blue method. Trypan blue (final concentration of 0.01% wt/vol; Sigma Chemical Co.) was added to each experimental group. Thereafter, aliquots of 10 µL were taken, and macrophages were counted. Morphologic evaluation of viable cells was performed by light microscopy (Olympus CKX 41) at 430X original magnification. More than 99% cells were viable of adherence purified macrophages before and upon incubation with and without autologous Hsp70.

$$\% \text{ cell Viability} = \frac{\text{Total number of viable cells}}{\text{Total number of cells}} X\ 100$$

Immunocytochemistry

Cytospin slides were prepared from normal peritoneal resident macrophage (NMO) and TAMs. Both groups of cells were incubated in medium with or without PMA and Hsp70-peptide for 24 hrs of time in RPMI 1640 containing 10% FCS, at 37°C in 5% CO_2 in humidified CO_2 incubator. Cells were fixed in 4% formaldehyde and permeablized with PBS buffer containing 0.02% Triton X-100 (Mahban *et al.*,(19). Then anti CD54-FITC antibody (1:100) was added and incubation was continued overnight at 4°C, followed by washing in PBS thrice. The fixed cells were stained with 10 mM/ml of Hoechst 33258, a kind of blue fluorescent dye (excitation/emission maxima~350/461 nm) used commonly for labeling nuclei respectively. After three washes with ice cold sterile PBS, cells were mounted in DABCO. The images were visualized with a Nikon E800 upright fluorescence microscope equipped Hoechst 33258 and FITC and PE filter with EXI aqua camera and NIS element software.

Flowcytometry

Cells were harvested from normal peritoneal resident macrophage (NMO) and TAMs. Both groups of cells were incubated in medium with or without PMA and auto Hsp70 for 24 hrs of time in RPMI 1640 containing 10% FCS, at 37°C in 5% CO_2 in humidified CO_2 incubator. Macrophages were suspended in RPMI 1640 with 10% FCS, 0.1 % NaN_3 and incubated (Xiuling et al.,) [20] with anti CD54 (ICAM-1) conjugated with FITC and anti CD14 conjugated with PE and isotype use as a control conjugated with FITC as per manufacture instruction. After washing, the cells were suspended in 0.1% PBS, and 0.1 %NaN_3 and then analyzed with a flowcytometry (BD Biosciences, Mountain View, CA, USA) equipped with an Innovate 90-5 (Coherent, Palo

Alto, CA, USA) argon ion laser operating at 488 nm and 515 mw in light regulated mode. Light scattering data and fluorescence parameter were collected by user-defined protocol and stored in list mode via lysis II program.

Western blotting

The protein lysate from cells of control and treated with PMA and Hsp70 was prepared in RIPA buffer and centrifuged at 10,000g for 15 min at 4°C. The supernatant was collected and protein concentration was determined by Bradford method (Bradford *et al.*, 1976). The cytosolic proteins (6µg/lane) were separated by 12% SDS-PAGE. Proteins were then transferred to PVDF membrane (Lilin *et al.*, 2008). The membrane was blocked in 5% (w/v) fat free skimmed milk, incubated for 2 h at 4°C washed in 1x PBS, incubated for overnight at 4°C and immunoblotted with a mouse anti-ICAM-1 monoclonal antibody followed by incubation with alkaline phosphatase conjugated antibody (Bangalore Genie, India) at a dilution of 1:5000.

RNA isolation

Total RNA was extracted from the MΦ and TAMs harvested from normal and DL bearing mice of the control and experimental mice groups with TRI reagent as instructed by the manufacturer. High quality RNA (as estimated by absorbance ratio A260/280P1.8) from different groups were resolved on 1% agarose gel and stained with ethidium bromide to check the integrity of 18S and 28S rRNA using UV transilluminator.

Detection of ICAM-1 mRNA by RT-PCR

RNA (5 lg) from each group of mice was first reverse transcribed into cDNA using reverse transcriptase. The resulting cDNA was used as a template for PCR amplification using specific primers for CD54 (ICAM-) and β- actin as an internal control. Primer sequence and PCR conditions are mentioned in Table 1. A typical 20-µL PCR (Xiuling et al.,) (20)contained 20 mM ammonium sulphate, 75 mM Tris-HCl, pH 8.8, 0.01% (vol/vol) Tween-20, 1 µM each primer, 2 µL of cDNA, 100 µM dNTPs, (Finnzyme, Biolabs, New England) 0.1% (wt/vol) BSA, and 0.25 U Taq polymerase (Genai, Banglore, India) and the following programme was used for reactions: 94°C for 3 minutes, 24 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute 30 seconds using Bio-Red MJ mini thermal cycler. PCR products were analysed by electrophoresis using a 2 % (wt/vol) agarose gel stained with ethidium bromide, and the intensity of each band was measured under UV fluorescence using Image analysis software form Gel documentation system (Bio-Red). The ratio of intensities of the bands for the gene product compared with the housekeeping gene β-actin was calculated and compared.

Table 1 Primer sequences and RT-PCR conditions

Gene	Primer Sequence	Amplicon Length	Annealing Temperature	No. of Cycle
CD54	FP 5'-TGG TAG CAG CCG CAG TCA TA-3'		68.8 °C	30
	RP 5'-CTC CTT CCT CTT GGC TTA GT-3'	281-400bp	59.1 °C	30
β- actin	FP 5'-ATC CAC GAA ACT ACC TTCAA-3'		58°C	30
	RP 5'-ATC CAC ACG GAG TAC TTG C-3'	300bp	60°C	30

Statistical analysis

Each value represents the mean SE_M of three independent experiments in each group except for in vivo stimulation experiments where two independent experiments were conducted. Data are analyzed by using two-tailed student's t-test on statistical software package Sigma Plot, version 12.0. Pearson's correlation coefficient was calculated for describing the colocalization correlation of the intensity distributions between two channels as (Kumar *et al.*, 2007) previously described. In each quantitative experiment with cells, 15 cells in total were analyzed. A value of $p < 0.05$ was considered significant.

RESULTS

Characterization of autologous Hsp70 and peritoneal macrophages

To determine whether purified peritoneal excaudate cells obtained from normal healthy and DL-bearing mice were macrophages, non-specific esterase staining of purified PECs were performed and positive red brown color formation (Fig. 2a) in more than 98 % cells in homologous cell preparation was observed. Further, cells were characterized by CD14-PE staining which indicates that more than 98% cells are macrophages (Fig. 2b).

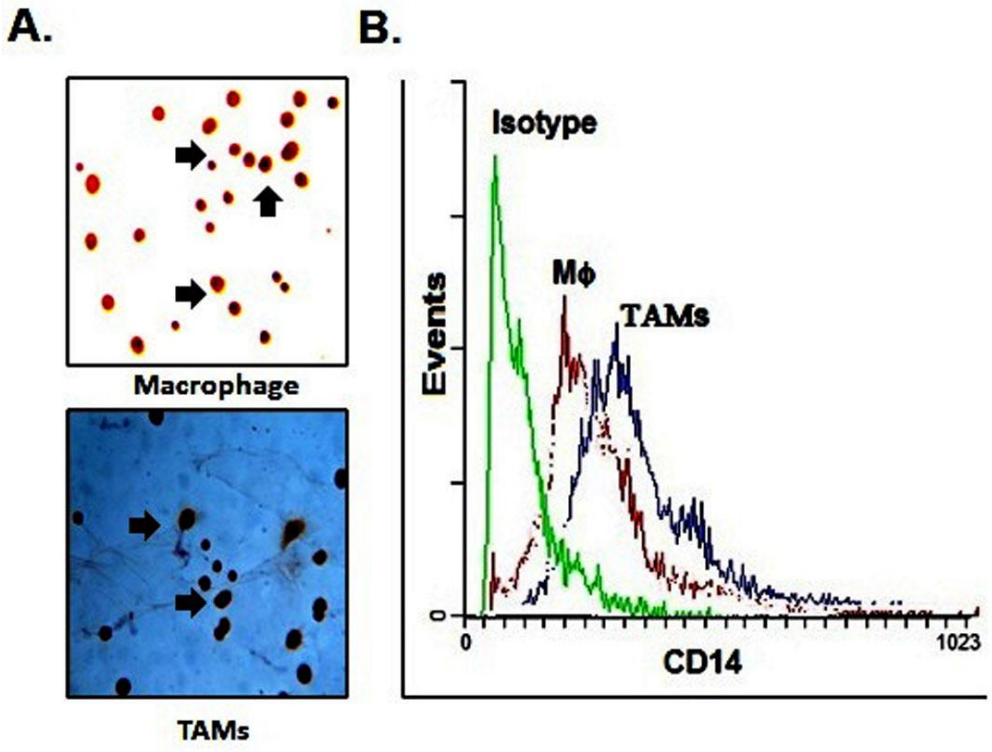

Fig. 2. Identification and Characterization of peritoneal macrophages. *MΦs were isolated from peritoneal exudates cells of normal healthy mice by adherence purification. Cells were counted and cultured in medium and divided into different groups for NSE staining and flowcytometric analysis. Cells*

were stained with α-naphthyl acetate and hexazotized pararosanaline as coupling agent which satins esterase in the cells non-specifically and viewed under inverted light microscope at different magnifications. Figure (a) shows Normal resident macrophages in upper panel while in lower panel shows TAMs with positive nonspecific esterase staining is seen as red-brown color in the cytoplasm of cells while Figure (B) shows flowcytometric confirmation of purified PECs from both the group were incubated with PE conjugated CD14 antibody. The percentage of $CD14^+$ positive staining cells in PECs was 98.00%. The Red line shows the $CD14^+$ cell of M1 phenotype (NMO), blue line shows M2 phenotype (TAMs) while green line shows Isotype control. The experiments were performed in triplicate. (Bar at 40X of figure A).

Homologous preparations of Hsp70 were purified from DL cell lysate in Sephadex column followed by DEAE-Sephacel column as described in Material and methods. The protein content of elute was measured by bradford method and 6 µg per lane of Hsp70 preparations were separated by SDS-PAGE. The Hsp70 purity is estimated to be greater than 95 % in this preparation as determined by bands obtained in staining (Fig 1) of SDS-PAGE of purified samples. The bands obtained were also checked and confirmed by immunoblots using Hsp70-specific monoclonal antibody.

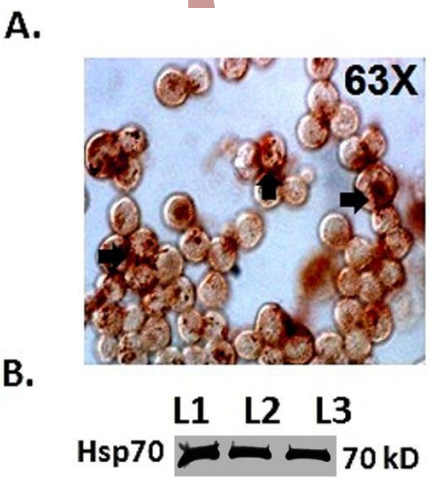

Fig. 1. Isolation and purification of hsp70-peptide. *PECs harvested from normal healthy mice, adherence purified, fixed in 4 % paraformaldehyde, permeabilized with 0.1 % Triton X-100 and stained with anti-Hsp70 for 2 hr then incubate with secondary antibody and visualized under dye. Immunocytochemical localization on the DL cell and immunoblotting of Hsp70. DL cells were lysed in lysis buffer and purified from sephadex G75 column followed by ADP/ATP agarose column. The protein concentration is estimated by standard method and 20 µg of proteins from elute were resolved on 12% polyacrylamide SDS gel, bands were transferred to PVDF membrane and incubated with anti-mouse Hsp70 antibody. Figure (A) Shows expression of expression of Hsp 70 on DL cell harvested from tumor bearing mice as a PECs while (B) immunoblot of Hsp70 eluted and purified mentioned in material and method, Lane 1, Lane 2 and Lane 3 show different elutes of Hsp70 upon purification after different samples. The experiments were performed in triplicate. (Bar at 630X).*

Expression of ICAM-1 by Immunofluresence

NMO and TAMs isolated from different group of mice were incubated with medium alone or medium containing Hsp70-peptide complex and expression of adhesion receptor CD54 (ICAM-1) expression was observed. It was observed that normal resident macrophages treated with Hsp70 resulted in significant increase in the expression of ICAM-1 receptor as compared to the normal resident macrophages incubated in medium alone. which is corresponding to (P<0.05) the observation that Hsp70 treatment resulted in increase in the (Fig. 3) tendency of macrophages while and it was observed that TAMs treated with Hsp70 resulted in significant increase in the expression of ICAM-1 receptor compared to the TAMs alone, which is corresponding to (P<0.05) the observation that hs70 treatment resulted in increase in the tendency of TAMs. Correspondingly, compare with the tams without incubation shows decreased receptor expression as compare to normal macrophages.

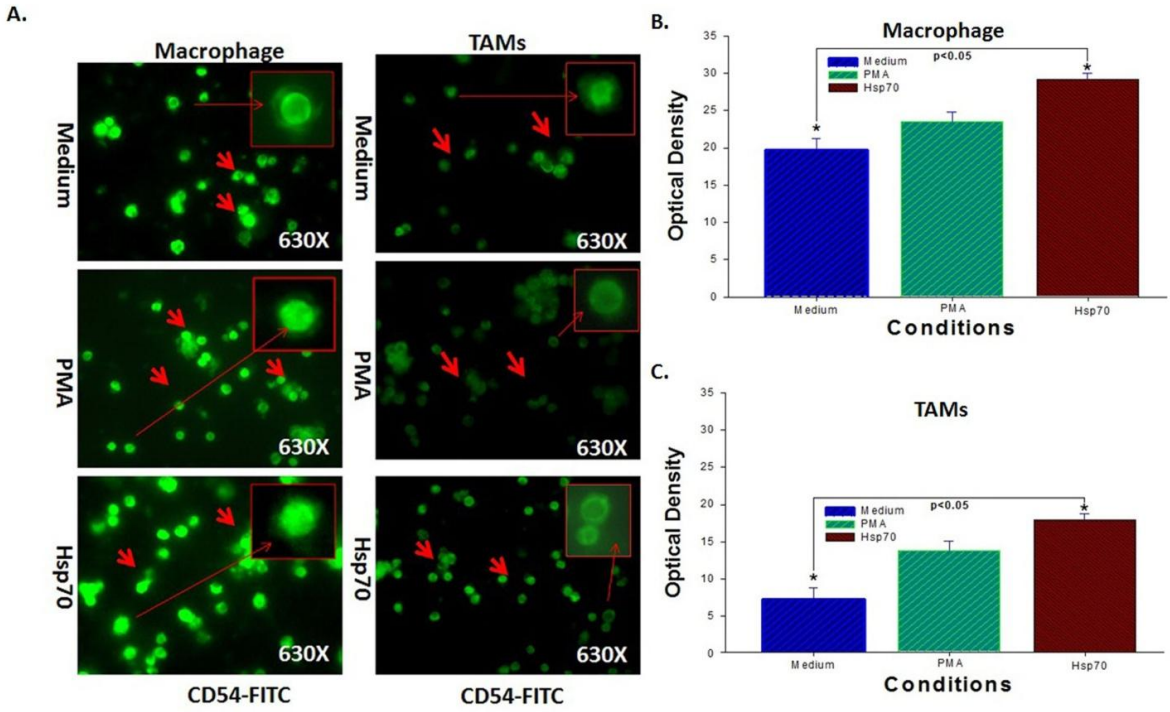

Fig. 3. Immunocytochemical localization of ICAM-1 in the macrophages. *MΦs were isolated from normal healthy and DL-bearing mice by adherence purification. MΦ and TAMs were incubated in medium with or without PMA or Hsp70 for 24 hrs. Harvested cells collected in RPMI supplemented with 10% FCS, cells were fixed in 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 and double stained with ICAM-1 conjugated FITC. Representative images showed the co-localization of ICAM-1 - FITC (green) in PECs form NH and TBH. (A) Showing macrophage with and without incubation of Hsp70, showing TAMs with and without incubation of Hsp70 and B and C showing expression in Bar graph of optical density. The experiments were performed in triplicate. (Bar at 630X)*

Expression of ICAM-1by Flowcytometry

Experiments were conducted to examining the effect of Hsp70 mediated receptor expression in NMO and TAMs harvested from different group of mice, cells were incubated in

medium and with or without PMA and Hsp70 peptide complex and expression of adhesion receptor CD54 (ICAM-1) was observed. it was found that normal resident macrophages treated with Hsp70 resulted in significant increase (169±13.01in NMO) and in the expression of ICAM-1 receptor as compared to the normal resident macrophages (34.1±3.04 for NMO with medium only)incubated in medium with or without PMA activation, which is corresponding to (P<0.05) the observation that Hsp70 treatment resulted in increase in the tendency of macrophages while Hsp70-mediated docking receptor expression in tumor associated macrophages (TAMs) it was observed that TAMs treated with Hsp70(Fig. 4) resulted in significant increase (30.1±3.1) in the expression of ICAM-1 receptor compared to the TAMs alone (9.0±1.23), which is corresponding to (P<0.05) the observation that hs70 treatment resulted in increase in the tendency of TAMs.

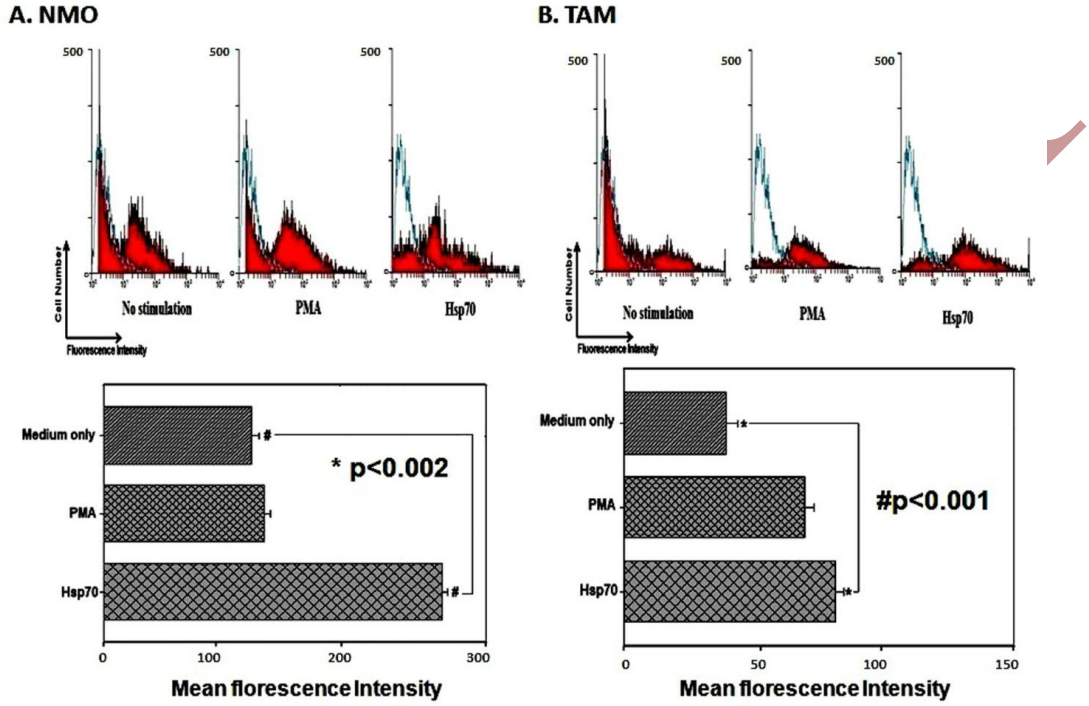

Fig. 4. Flowcytometric analysis of expression of CD54 molecule in phenotype of macrophages. *MΦs were isolated from normal healthy and DL-bearing mice and adherence purified, MΦs and tams were incubated in medium with or without PMA or hsp70 for 24 hrs. Cells were double stained with CD14-PE as a cell marker, CD54-FITC and Isotype control-FITC to detect distribution of CD54 on cytoplasm of phenotype. (a) Showing the Normal peritoneal macrophages while (b), showing Peritoneal tumor associated macrophages harvested (tams) from tumor bearing host, found enhanced CD54 expression upon incubation with hsp70. Each experiment performs in triplicates. Each bar represents the mean ± SEM of 3 independent experiment. # p < 0.05 compared to medium and incubation of cells with hsp70, *p < 0.05 medium and incubation of TAMs with hsp70.*

Expression of ICAM-1by Western Blotting

Western blot analysis of adhesion receptor CD54 (ICAM-1) expression in TAMs shows a band with an estimated molecular weight of <50kDa, (known molecular mass of protein)

respectively. Result shows that Hsp70 incubation increases the expression level of adhesion and (Fig. 5) fusion protein in NMO and TAMs. TAMs without incubation (medium lone) show basal level of Protein expression while NMO with medium only shows sharp enhances expression a compare to TAMs.

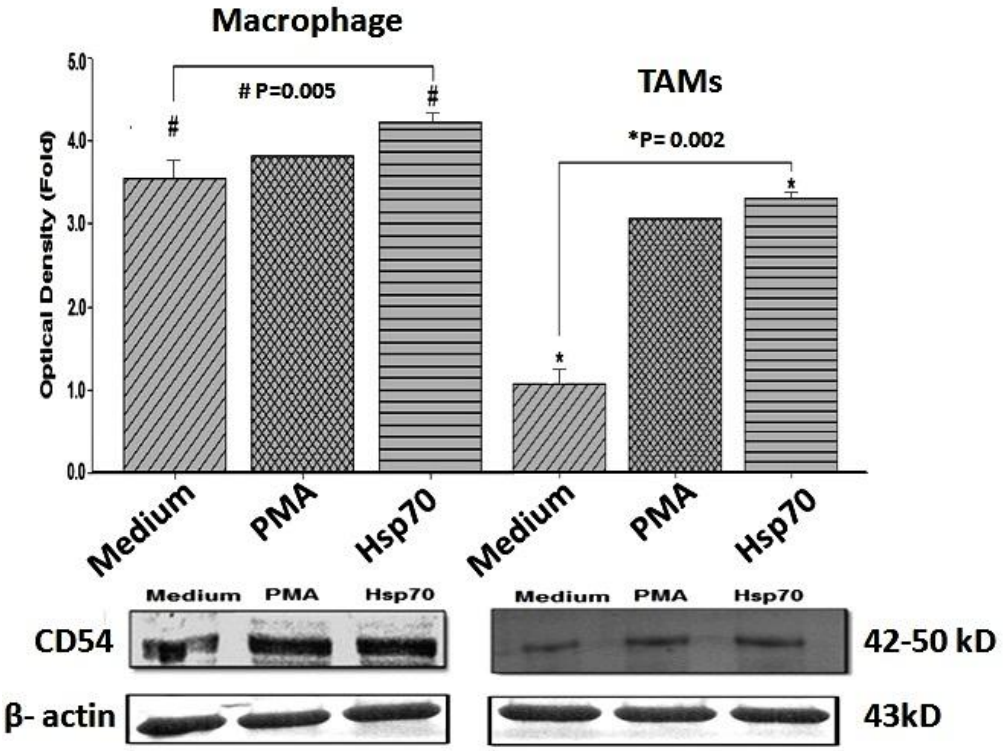

Fig.5. Effect of Hsp70 treatment on CD54 protein expression of macrophages. *Peritoneal MΦs harvested from normal healthy mice treated with medium alone or medium containing hsp70 were recovered and after 24 hours of incubation, Purified fraction of cells were lased in RIPA buffer and analyzed by 12% SDS-PAGE with marker and band were transfer to PVDF membrane, bands obtained were incubated with anti- CD54 specific monoclonal antibody and color was developed as described in Materials and Methods. Data is calculated as the ratio of integrated densitometric values (IDV) of CD54 to β- actin content in each group and plotted as relative percentage units. Each bar represents the mean ± SEM of 3 mice. Each bar represents the mean ± SEM of 3 independent experiments. # p < 0.05 compared to medium and incubation of cells with Hsp70, *p < 0.002 medium and incubation of TAMs with Hsp70.*

Expression of ICAM-1by RT-PCR

Experiments were conducted to examining the effect of Hsp70 mediated docking receptor expression in NMO and TAMs harvested from different group of mice, cells were incubated in medium and with or without PMA and Hsp70-peptide complex, expression of adhesion receptor CD54 was observed. it was found that normal resident macrophages treated with Hsp70 resulted in significant increase(14%) and in the expression of ICAM-1 receptor as (Fig. 6) compared to the normal resident macrophages incubated in medium with or without PMA activation, which is corresponding to (P<0.05) the observation that Hsp70 treatment resulted in increase in the

tendency of macrophages while Hsp70-mediated docking receptor expression in tumor associated macrophages (TAMs) it was observed that TAMs treated with Hsp70 resulted in significant increase (245%) in the expression of ICAM-1 receptor compared to the TAMs alone, which is corresponding to ($P<0.05$) the observation that hs70 treatment resulted in increase in the tendency of TAMs.

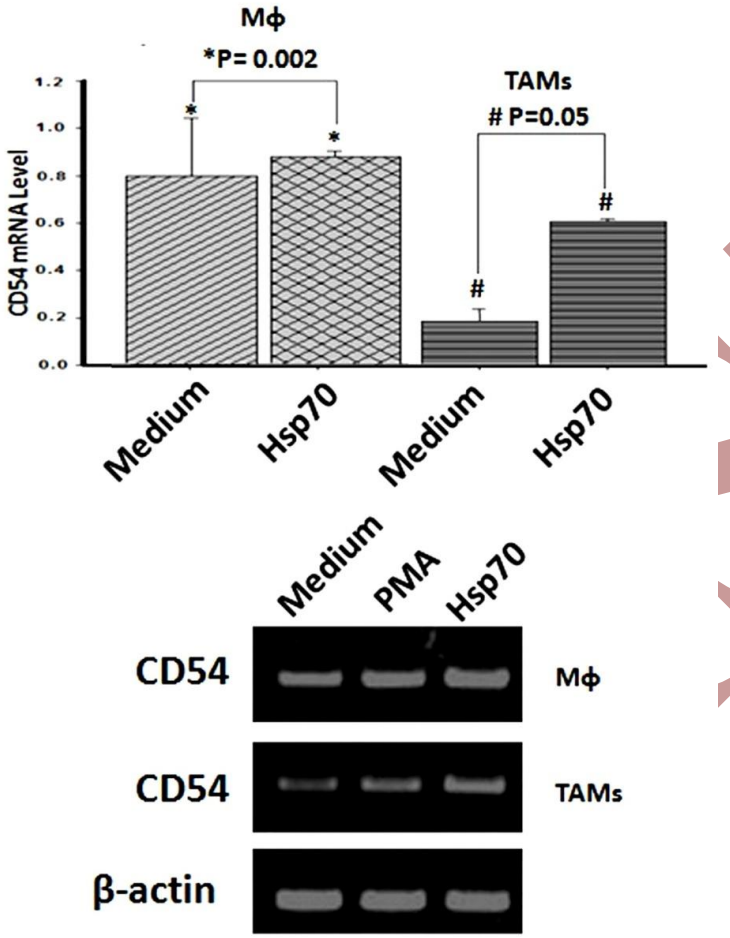

Fig.6. Effect of Hsp70 treatment on CD54 mRNA expression of macrophages. *MΦs were isolated from normal healthy and DL-bearing mice and adherence purified, macrophages and tams were incubated in medium with or without PMA or hsp70 for 24 hrs and total RNA were collect as described in Materials and Methods. Data is calculated as the ratio of integrated densitometry values (IDV) of CD54 mRNA and β- actin in each group and plotted as relative percentage units. Each bar represents the mean ± SEM of 3 independent experiments. # $p < 0.05$ compared to medium and incubation of cells with Hsp70, *$p < 0.05$ medium and incubation of TAMs with Hsp70*

DISCUSSION

In the present study, we used autologous Hsp70-peptide complex purified from Dalton's lymphoma cells (Figure 1a) and determined its effect on the expression of docking receptor (ICAM-1) in TAMs harvested from DL bearing mice. Our data demonstrated the (i) expression level of ICAM-l in Mϕ and TAMs in control group and (ii) its increased expression in autologous Hsp70-peptide complex treated Mϕ and TAMs; suggested the direct effect of autologous Hsp70-peptide complex on Mϕ and TAMs in the production of anti-tumor cytokines and lamellipodia formation similar to previous observation (Kumar *et al.,* 2006; Gautam et al., 2013). Multiple and various cell adhesion molecules take part in intercellular and cell to extracellular matrix interactions with tumor. Tumor progression is a multistep process in which adhesion molecules play an essential role in the development of recurrent (Table 2) invasiveness and distant metastatic proportion of tumor cells (1).

Table 2 CD54 (ICAM-1) receptor expression on macrophage phenotypes

	Immuncytochemistry					
	Macrophages			TAMs		
	Medium only (Control)	PMA	Hsp70	Medium only (Control)	PMA	Hsp70
Increases of mean value CD54	-X	*	1.5X	-X		1.6X
R and P value Med and Hsp70		R=0.125 P<0.05			R=0.014 P<0.005	
	Flowcytometry					
Increases of mean value CD54	-X (Control)		2.15X	-X	2.4X	2.2X
P value Med and Hsp70		P<0.05			P<0.005	
	Immunoblotting					
Increases of mean value CD54	-X	2.01X	2.23X	-X	1.15X	1.39X
P value Med and Hsp70		P<0.05			P<0.005	
	RT –PCR					
Increases of mean value CD54	-X	*	1.1X	-X	*	4.21X
P value Med and Hsp70		P<0.005			P<0.002	

*ND= Not defined, R= Pearson's correlation coefficient(+1 to-1), P= Unpaired Student t test (≤1)

A growing body of evidence indicates that alterations in the adhesion properties of neoplastic cells play a pivotal role in the development and progression of cancer. Loss of intercellular

adhesion and the desquamation of cells from the underlying lamina propria allow malignant cells to escape from their site of origin, extracellular matrix, and acquire a more motile and invasive phenotype (Maio *et al.*, 1992; Makgoba *et al.*, 1989). By comparing the data with, it was found that tumor microenvironment suppresses the anti-tumor property of MΦ and TAMs convert then from phenotypically more active (M1) MΦ to phenotypically less active (M2) which rather support tumor progression.

It was found the expression of docking ICAM-1 expression was drastically decrease (14.1±9.1) on TAMs as compare to normal resident macrophages (78± 12.02) which facilitate the tumor progression by loss of cell-cell contact between (Apc-Icam-1:T cell-Lfa-1). As a result interaction between other molecules necessary for T cell response has lost and cause T cell anergy. Moreover, it has been shown that loss of ICAM-1 expression inhibits the transmigration of Mϕ and TAMs to the tumor site that inhibit anti tumor activity of tumor resident macrophages (Mϕ and TAMs). In addition to participating in tumor invasiveness and metastasis, adhesion molecules also regulate or significantly contribute to a variety of functions including signal transduction, cell growth, differentiation, site-specific gene expression, morphogenesis, immunologic function, cell motility, wound healing, and inflammation (Fig. 7).

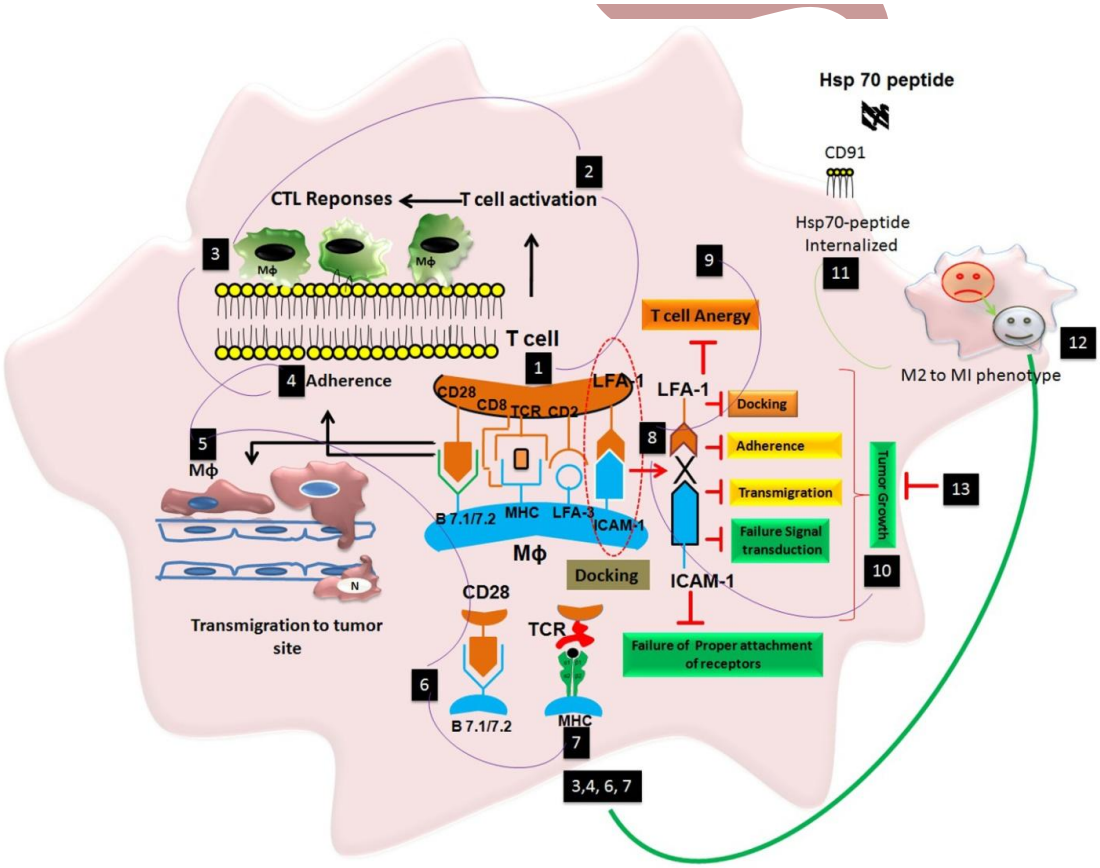

Fig.7. Putative model for correlating the modulation of CD54 expression level with Hsp70changes in TAMs. *Cartoon showing the possible correlation of the effect of tumor progression on CD54 expression on Mϕ and Tams. This cartoon shows that he possible role of ICAM-1 in the presence and absence over APC cells. ICAM-1 expressed over APCs strongly MΦ and it legend expressed over T cells that facilitate docking between T cell and MΦ (1) for further interaction of other receptors (6, 7) for immune responses such as T cell activation and CTL responses (2), Migration(3), Adherence(4) and transmigration to the site of tumor (5). During tumor progression the expression of ICAM-1 impaired and instead of activation cell become stressed and immune function such as docking, adherence, migration, blocked results T cell undergo anegic state (9) instead of activation which support tumor progression (10). During coincubation of suppressed TAMs with Hsp70 peptide complex, it interact and internalized via CD91 receptor expressed (11) over cells facilitate the transformation of M2 state of MΦ into M1 state (12) of activated state that facilitate regression of tumor growth (13).*

**Many other molecules are also involved for the receptor expression but this cartoon includes those molecules relevant to Hsp70 induced signaling cascade.*

Our study showed basal level of ICAM-1 expression in TAMs was enhances upon incubation with Hsp70-peptide complex (31.01±2.23) but it was at lesser extent as compare to NMO (Figure 6). As a result Hsp70-peptide complex revert the suppressed property of TAMs (M2) to slightly M1 activated state that fight against tumor progression by releasing various anti-tumor cytokines and surface expressions of co-stimulatory molecules.

The results from the prior studies, combined with our new findings, suggest the hypothesis that suppressed function of alternatively activated macrophages can be regained by the treatment of autologous Hsp70-peptide complex and led to enhanced production of cytokines, chemokines, and their receptors, RNI and ROI(Gautam *et al.*, 2013). Our study indicated that Hsp70- peptide complex treatment results in significant changes in morphological profile of TAMs that are comparable to that of classically activated macrophages. Mϕ incubated with Hsp70- peptide complex for 24 h showed flattened shape, increased number of narrow, elongated lamellipodia and enhanced adherence as compared to Mϕs incubated in medium only (Lucimara et al., 2008; Gautam et al., 2013). On contrary, TAMs incubated in culture medium only for 24 hrs showed oval shape morphology and minute expression of lamellipodia as compare to Mϕ harvested from normal healthy mice without any activation of Hsp70 or PMA indicating that exogenous application of Hsp70 results in the overall transformation of M2 phenotype to M1 phenotype in terms of both morphology and anti-tumor function (Boyd *et al.*, 1998). Moreover, it was also found that hsp70-peptide enhances the expression of fusion receptor CD172a and CD47, MHC class I expression in Mϕ and TAMs facilitate tumor regression (Gautam *et al.*, 2014; Gautam *et al.*, 2015).

In a nut shell, it can be concluded from our observations that exogenous application of tumor derived Hsp70 to peritoneal macrophages *in vitro* leads to the enhanced expression of ICAM-1 (CD54) that enhances the docking between T cell for proper cell to cell interaction and further interaction of other receptors. Moreover, ICAM-1 (CD54) significantly contribute to a

variety of functions including signal transduction, cell growth, differentiation, site-specific gene expression, morphogenesis, cell motility, wound healing, and inflammation take one more step to support that this molecule act against cancer progression and can be effectively utilized in immunotherapy and vaccination.

CONFLICT OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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FIGURE LEGEND

Fig. 1. Isolation and purification of hsp70-peptide. PECs harvested from normal healthy mice, adherence purified, fixed in 4 % paraformaldehyde, permeabilized with 0.1 % Triton X-100 and stained with anti-Hsp70 for 2 hr then incubate with secondary antibody and visualized under dye. Immunocytochemical localization on the DL cell and immunoblotting of Hsp70. DL cells were lysed in lysis buffer and purified from sephadex G75 column followed by ADP/ATP agarose column. The protein concentration is estimated by standard method and 20 µg of proteins from elute were resolved on 12% polyacrylamide SDS gel, bands were transferred to PVDF membrane and incubated with anti-mouse Hsp70 antibody. Figure (A) Shows expression of expression of Hsp 70 on DL cell harvested from tumor bearing mice as a PECs while (B) immunoblot of Hsp70 eluted and purified mentioned in material and method, Lane 1, Lane 2 and Lane 3 show different elutes of Hsp70 upon purification after different samples. The experiments were performed in triplicate. (Bar at 630X).

Fig. 2. Identification and Characterization of peritoneal macrophages. MΦs were isolated from peritoneal exudates cells of normal healthy mice by adherence purification. Cells were counted and cultured in medium and divided into different groups for NSE staining and flowcytometric analysis. Cells were stained with α-naphthyl acetate and hexazotized pararosanaline as coupling agent which satins esterase in the cells non-specifically and viewed under inverted light microscope at different

magnifications. Figure (a) shows Normal resident macrophages in upper panel while in lower panel shows TAMs with positive nonspecific esterase staining is seen as red-brown color in the cytoplasm of cells while Figure (B) shows flowcytometric confirmation of purified PECs from both the group were incubated with PE conjugated CD14 antibody. The percentage of $CD14^+$ positive staining cells in PECs was 98.00%. The Red line shows the $CD14^+$ cell of M1 phenotype (NMO), blue line shows M2 phenotype (TAMs) while green line shows Isotype control. The experiments were performed in triplicate. (Bar at 40X of figure A).

Fig. 3. Immunocytochemical localization of ICAM-1 in the macrophages. MΦs were isolated from normal healthy and DL-bearing mice by adherence purification. MΦ and TAMs were incubated in medium with or without PMA or Hsp70 for 24 hrs. Harvested cells collected in RPMI supplemented with 10% FCS, cells were fixed in 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 and double stained with ICAM-1 conjugated FITC. Representative images showed the co-localization of ICAM-1 - FITC (green) in PECs form NH and TBH. (A) Showing macrophage with and without incubation of Hsp70, showing TAMs with and without incubation of Hsp70 and B and C showing expression in Bar graph of optical density. The experiments were performed in triplicate. (Bar at 430X)

Fig. 4. Flowcytometric analysis of expression of CD54 molecule in phenotype of macrophages. MΦs were isolated from normal healthy and DL-bearing mice and adherence purified, MΦs and tams were incubated in medium with or without PMA or hsp70 for 24 hrs. Cells were double stained with CD14-PE as a cell marker, CD54-FITC and Isotype control-FITC to detect distribution of CD54 on cytoplasm of phenotype. (a) Showing the Normal peritoneal macrophages while (b), showing Peritoneal tumor associated macrophages harvested (tams) from tumor bearing host, found enhanced CD54 expression upon incubation with hsp70. Each experiment performs in triplicates. Each bar represents the mean ± SEM of 3 independent experiment. # $p < 0.05$ compared to medium and incubation of cells with hsp70, *$p < 0.05$ medium and incubation of TAMs with hsp70.

Fig.5. Effect of Hsp70 treatment on CD54 protein expression of macrophages. Peritoneal MΦs harvested from normal healthy mice treated with medium alone or medium containing hsp70 were recovered and after 24 hours of incubation, Purified fraction of cells were lased in RIPA buffer and analyzed by 12% SDS-PAGE with marker and band were transfer to PVDF membrane, bands obtained were incubated with anti- CD54 specific monoclonal antibody and color was developed as described in Materials and Methods. Data is calculated as the ratio of integrated densitometric values (IDV) of CD54 to β- actin content in each group and plotted as relative percentage units. Each bar represents the mean ± SEM of 3 mice. Each bar represents the mean ± SEM of 3 independent experiments. # $p < 0.05$ compared to medium and incubation of cells with Hsp70, *$p < 0.002$ medium and incubation of TAMs with Hsp70.

Fig.6. Effect of Hsp70 treatment on CD54 mRNA expression of macrophages. MΦs were isolated from normal healthy and DL-bearing mice and adherence purified, macrophages and tams were incubated in medium with or without PMA or hsp70 for 24 hrs and total RNA were collect as described in Materials and Methods. Data is calculated as the ratio of integrated densitometry values (IDV) of CD54 mRNA and β- actin in each group and plotted as relative percentage units. Each bar represents the mean ± SEM of 3 independent experiments. # $p < 0.05$ compared to medium and incubation of cells with Hsp70, *$p < 0.05$ medium and incubation of TAMs with Hsp70.

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*Many other molecules are also involved for the receptor expression but this cartoon includes those molecules relevant to Hsp70 induced signaling cascade.

Table 1 Primer sequences and RT-PCR conditions

Gene	Primer Sequence	Amplicon Length	Annealing Temperature	No. of Cycle
CD54	FP 5'-TGG TAG CAG CCG CAG TCA TA-3'		68.8 °C	30
	RP 5'-CTC CTT CCT CTT GGC TTA GT-3'	281-400bp	59.1 °C	30
β- actin	FP 5'-ATC CAC GAA ACT ACC TTCAA-3'		58°C	30
	RP 5'-ATC CAC ACG GAG TAC TTG C-3'	300bp	60°C	30

Table 2 CD54 (ICAM-1) receptor expression on macrophage phenotypes

	Immuncytochemistry					
	Macrophages			TAMs		
	Medium only (Control)	PMA	Hsp70	Medium only (Control)	PMA	Hsp70
Increases of mean value CD54	-X	*	1.5X	-X		1.6X
R and P value Med and Hsp70		R=0.125 P<0.05			R=0.014 P<0.005	
	Flowcytometry					
Increases of mean value CD54	-X (Control)		2.15X	-X	2.4X	2.2X
P value Med and Hsp70		P<0.05			P<0.005	
	Immunoblotting					
Increases of mean value CD54	-X	2.01X	2.23X	-X	1.15X	1.39X
P value Med and Hsp70		P<0.05			P<0.005	
	RT –PCR					
Increases of mean value CD54	-X	*	1.1X	-X	*	4.21X
P value Med and Hsp70		P<0.005			P<0.002	

*ND= Not defined, R= Pearson's correlation coefficient(+1 to-1), P= Unpaired Student t test (≤1)