SUPPRESSED EXPRESSION OF CD80 (B7.1) AND CD86 (B7.2) RECEPTORS IN TAMS UP-REGULATED BY AUTOLOGOUS HSP70– PEPTIDE COMPLEX IN DALTON'S LYMPHOMA BEARING BALB/C MICE

***Pramod Kumar Gautam, A. Acharya**

**Department of zoology, Faculty of Science, Banaras Hindu University, Varanasi-221005, U.P., India*

ABSTRACT

Co-stimulatory molecules (CSMs) are member of immunoglobulin (Ig) superfamily receptors which expressed on macrophages. CSMs play important role in the stimulation of cell mediated immune responses and play important role in the activation of T cells. Activation of T cells is thought to require two different signals. The first signal (recognition) requires interaction of the T cell receptor with an antigen bound Major histocompatibility complex (MHC) and the second signal is provided by the CD80 or CD86 on the antigen presenting cell and CD28 and CTLA-4 on the reacting T cell. In the aberration of proper co-stimulatory signal, the T cell undergoes anergy state instead of activation which favors the tumor progression. During tumor progression, tumor microenvironment downregulate the expression of CSMs in M1 phenotype and function and transform into M2 phenotype facilitate tumor progression. Heat shock proteins 70 (Hsp70) are highly conserved group of cytosolic protein which have important role in growth, homeostasis. Hsp70 is activator of macrophages and enhances the release of specific and nonspecific effector molecules that have major role in tumor destruction and immunopotentiation of host. It has been observed that Hsp70 preparations derived from normal tissues do not elicit tumor immunity, while Hsp70 preparation derived from tumor cell able to elicit tumor immunity. However, this study examined the expression of CD80 and CD86 along with Hsp70-peptide complex over the tumor infiltrating TAMs to assess their significance in Leukemia. Study shows that a remarkable decline in CSMs expression in M2 phenotypes, which was recovered after Hsp70-peptide complex treatment. These findings suggest that CSMs might be involved in tumor progression when their expression down regulated while Hsp70-peptide treatment enhances the expression in TAMs that facilitate tumor regression by activating T cell.

Keywords: Co-stimulatory molecules: TAMs: Tumor; Macrophages: Hsp70 peptide complex

Abbreviation: NMO: Normal Peritoneal Macrophages; TAMs: Tumor Associated Macrophages; NH: Normal Healthy Mice; TBH: Tumor Bearing Host; DL: Dalton's lymphoma; APC: Antigen Presenting Cell; CSMs: Co-stimulatory molecules; MMP: Matrix Metallo Proteases.

INTRODUCTION

Macrophages are mononuclear phagocytic fundamental cell of the innate defense mechanisms originate [1, 2], which can promote specific immunity by inducing T cell recruitment and activation. It acts as antigen-presenting cells and displays MHC class I and II bound antigen to T lymphocytes for effective generation of cancer cell specific cell-mediated immunity. T cell activation is thought to require two signals. The first signal (recognition) requires interaction of the T cell receptor with an antigen bound MHC and the second signal is provided by the CD80 or CD86 on the antigen presenting cell and CD28 and CTLA-4 on the reacting T cell [3, 4]. Two distinct B7 molecules such as B7.1 (CD80) and B7.2 (CD86), a transmembrane glycoprotein members [5-7] of the Immunoglobulin family expressed on antigen-presenting cells (APCs) with their T cell counter receptors CD28 (8) and CTLA-4 [9-11]. Interaction of B7 and CD28 has been shown to provide a critical signal for T cell activation, while absence produce T cell anergic state [12-14].

These cells are recruited into tumor microenvironment as monocyte from the bloodstream attracted by the graded chemoattractant which produced by tumor cells such as CCL2, vascular endothelial growth factor (VEGF), and CXCL12 (SDF1) gradient and converted into macrophages [15, 16]. Macrophages, which are found close vicinity to the site of tumor microenvironment, are known as tumor associated macrophages (TAMs) or M2 macrophages (alternatively activated) (17) which constitutes about 10-20% of total tumor mass [18, 19]. The type II macrophages or M2 macrophages are characterized by suppressed cytotoxic function, reduced surface receptor expression and enhanced production of anti-inflammatory cytokines that results in skewing of immune responses to Th2 type at the tumor microenvironment [20].

Several documents and avenues of communication are available about HSPs function; HSP70 is expressed constitutively as well as inducibly by stimulation protects cell from the subsequent damage [21]. Hsp70 is highly conserved and load with immunopotential capability inducing the activation of B and T cell. Hsp70 cross present tumor associated antigens (TAAs) via mhc 1 as well as mhc II molecules. It can activate both normal peritoneal resident MΦs and TAMs leading to enhanced production of NO, H_2O_2, inflammatory cytokines, chemokines, and cytokine and chemokine receptors in addition to its profound role in eliciting cancer-specific immunity against the tumor by virtue of their ability to bind tumor-specific peptides. Hsp70 purified from unstressed tissues do not produce tumor immunity, while Hsp70-peptides derived from tumor cell shown only due to antigenic peptides associated with it [22-24]. Moreover, immunity towards tumor elicited by immunization with HSP-peptide is mediated by $CD8^+$ T cells, and its mechanism involves MHC class I molecule restricted response [25].

Keeping the immunomodulatory function of Hsp70-peptide complex in mind, the expression of B7 molecules during tumor burden has not been investigated. Therefore, this study examined the

expression of B7 family of co-stimulatory molecules along with Hsp70-peptide complex over the tumor infiltrating TAMs to assess their significance in Dalton's lymphoma.

MATERIAL AND METHOD

Animals and tumor model

Inbreed populations of BALB/c ($H2^d$) 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*.

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.

Reagents

RPMI 1640 culture medium was obtained from HiMedia, Mumbai, India. FBS was obtained from Invitrogen, CA, USA and Goat serum was prepared and heat inactivated in the lab for plastic petridish coating to purify TAMs. Anti-hsp70 was obtained from Biolegand, San Diego, CA, USA. CD80, CD86 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. The ATP/ADP agarose, G-75 sephadex column, phorbol 12-myristate 13-acetate (PMA), hoechst 33258 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Na2HPO4, KH2PO4, formaldehyde and trypsin were purchased from Qualigens, Mumbai, India, acetone was purchased from Rankem Ltd., Mumbai, India, and glutaraldehyde was obtained from Serva Electrophoresis, Heidelberg, Germany. All other chemicals otherwise stated were obtained from Qualigens.

Isolation and purification of hsp70

The hsp70 were isolated and purified as previously described [26] 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). Eluates were 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. 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 hrs 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. 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 petridishes (Tarson, Kolkata, India) at 37°C in CO_2 incubator (Shelab, Oregon, CA, USA) for 2 hrs. 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 flask (Tarson, Kolkata, India) at a cell density of 1×10^6 in the culture medium with or without PMA and hsp70 and incubated for time periods of 24 hrs in CO_2 incubator.

Identification and characterization of macrophages

Adherent cells were washed in chilled PBS and fixed for 15 min using 0.2% glutaraldehyde in PBS. The cells were stained for 1 hrs 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. The reaction was stopped by removing the solution of α naphthyl butyrate-NaF and rinsing the cells with water. Cells were counted under light microscopy. Those cells that exhibited bright red, diffuse cytoplasmic staining were considered to be positive for nonspecific esterase. Adherent cells were also fixed in 4% paraformaldehyde, stained with Giemsa and examined with a phase-contrast microscope. Further, purified macrophages were confirmed by flowcytometric analysis by using specific $CD14^+$ antibody conjugated with PE flurochrome.

Cell viability Assay

Peritoneal exudates cells (PECs) were harvested by peritoneal lavage using chilled serum free culture medium RPMI 1640. The PECs were then transferred 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 of cells. Thereafter, aliquots of 10μL were taken and macrophages were counted in a hemocytometer chamber. Morphologic evaluation of viable cells was performed by light microscopy (Olympus CKX 41, Center Valley, PA, USA) at 430X original magnification.

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

Confocal microscopy

Cytospin slides were prepared from NMO and TAMs. Both the groups of cells were incubated in medium with or without PMA and hsp70 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% Paraformaldehyde and permeablized with PBS buffer containing 0.3% triton X-100. Then anti CD80 FITC and anti-CD86-FITC antibody (1:100) was added as per manufacturer instructions and incubation was continued overnight at 4°C, followed by washing in PBS. The fixed cells were stained with 10 mM/ml of hoechst 33258, a kind of blue fluorescent dye used commonly for labeling nuclei respectively. After three washes with ice cold sterile PBS, cells were mounted in DABCO. The images were visualized with Carl Zeiss confocal microscope, equipped Zen 2012 software under the central facility of ISLS, BHU, Varanasi, India.

Flowcytometry

Cells were prepared from NMO and TAMs. Both the groups of cells were incubated in medium with or without PMA and autologous 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 RPMI1640 with 10% FCS, 0.1 % NaN_3 and incubated with anti mouse CD80 and CD86 antibody conjugated with FITC and anti mouse CD14 conjugated with PE and isotype use as a control conjugated with FITC as per manufacture instruction. After washing, cells were suspended in 0.1% PBS containing 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 autologous 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. The purified proteins were separated by 12% SDS PAGE and proteins bands were then transferred onto PVDF membrane. The membrane was blocked in 5% (w/v) fat free skimmed milk, incubated for 2 hrs at 4°C washed in 1x PBS. PVDF membrane were incubated with a mouse anti CD80 and anti CD86 monoclonal antibody for overnight at 4°C followed by ALP conjugated antibody (Bangalore Genie, India) at a dilution of 1:100 for 2 hrs.

RNA isolation

Total RNA was extracted from NMO and TAMs harvested from normal healthy mice 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 1.2% agarose gel and stained with ethidium bromide (Etbr) to check the integrity of 18S and 28S rRNA using UV transilluminator.

Reverse transcriptase-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 CD80 (B 7.1), CD 86 (B 7.2) and β actin as an internal control. Primer sequence and PCR conditions are mentioned in Table 1. A typical 20μL PCR contained 20mM ammonium sulphate, 75mM Tris-HCl, pH 8.8, 0.01% (vol/vol) Tween 20, 1μM each primer, 2μL of cDNA, 100μM 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 analyzed by electrophoresis using a 1 % (wt/vol) agarose gel stained with ethidium bromide, and the intensity of each band was measured under UV fluorescence using image analysis software from gel documentation system (BioRed). 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
CD80 (B7.1)	FP 5'-CTGCTTGCTCAACTCTACGTC -3'	180-150	64 °C/62 °C	30
	RP 5'-CTGAAGTTAGCTTTGACTGATAACG -3'			
CD86 (B7.2)	FP 5'-GCAATAGCATCACAAATTTCA-3'	255-300	56 °C/64 °C	30
	RP 5′-TCAGTCAAAGCTAACTTCAGTCAACC -3′			
β- actin	FP 5'-ATC CAC GAA ACT ACC TTCAA-3'	300	58.7 °C/60.2 °C	30
	RP 5'-ATC CAC ACG GAG TAC TTG C-3'			

Table 2 B7 family receptor expression on macrophage phenotypes in different conditions

	Parameters	Macrophages			TAMs		
		Medium only	*PMA*	*Hsp70*	*Medium only*	*PMA*	*Hsp70*
Immuncytochemistry	Increases of mean value CD80	-X	1.71X	2.10X	-X	1.10	1.21X
	P value Med and Hsp70	*P<0.05*			*P<0.001*		
	Increases of mean value CD86	-X	1.90X	4.02X	-X	2.10X	3.5X
	P value Med and Hsp70	*P<0.007*			*P<0.002*		
Flowcytometry	Increases of mean value CD80	-X (Control)	1.02X	2.04X	-X	2.75X	5.61X
	P value Med and Hsp70	*P<0.05*			*P<0.001*		
	Increases of mean value CD86	-X	1.52X	2.32X	-X	3.97X	5.42X
	P value Med and Hsp70	*P<0.007*			*P<0.001*		
Immunoblotting	Increases of mean value CD80	-X	56%	66%	-X	106%	128%
	P value Med and Hsp70	*P<0.001*			*P<0.005*		
	Increases of mean value CD86	-X	63%	77.7%	-X	94%	156%
	P value Med and Hsp70	*P<0.001*			*P<0.005*		
RT -PCR	Increases of mean value CD80	-X	41%	55%	-X	123%	187%
	P value Med and Hsp70	*P<0.001*			*P<0.005*		
	Increases of mean value CD86	-X	20%	27.4%	-X	154%	176%
	P value Med and Hsp70	*P<0.005*			*P<0.001*		
P= Unpaired Student *t* test (≤1)							

Statistical analysis

Each value represents the mean SE_M of three independent experiments in each group except for *in vitro* 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. A value of $p < 0.05$ was considered significant.

RESULTS

Characterization and identification of peritoneal macrophage and hsp70.

Purified PECs obtained from normal healthy and DL bearing mice. Non specific esterase staining of purified PECs were performed and positive red brown color formation (Fig.1) and further cells were characterized by CD14 PE and Isotype conjugated with FITC staining which indicates that more than 98%±1.2 cells was found macrophages. Homologous preparations of hsp70 were confirmed by western blotting using hsp70-specific monoclonal antibodies (Fig. 2).

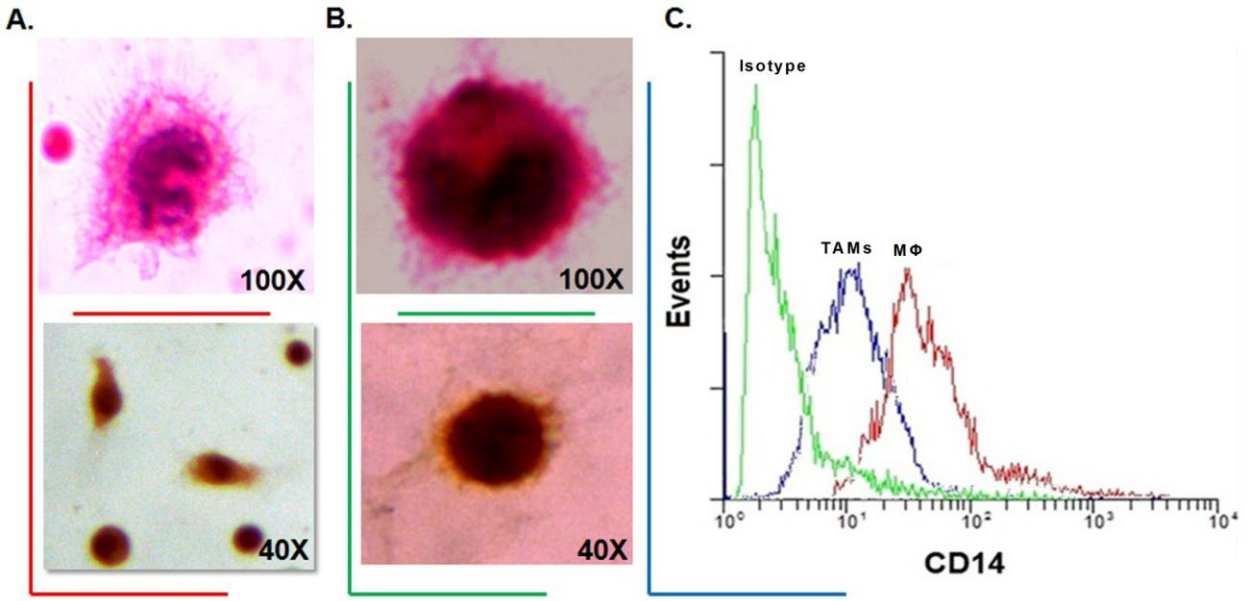

Fig. 1

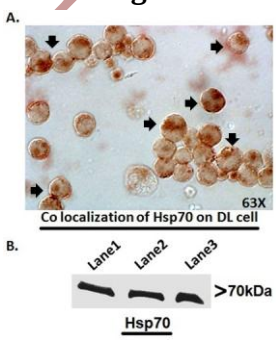

Fig. 2

Co-localization of CD80/CD86 expression on the Surface of macrophages

NMO and TAMs isolated from different groups of mice were incubated with medium alone or medium containing Hsp70, and expression of fusion receptor CD80 and CD86 was observed. It

was observed that normal resident macrophages treated with Hsp70 resulted in significant increase in the expression of CD80 receptor (150±34)and CD86 receptors (198± 45.1) as compared to the normal resident macrophages of CD80 (71.3± 12.2) and CD86 (53±9.23)incubated in medium only, which is corresponding to ($p < 0.05$) the observation that Hsp70 treatment resulted in increase in (Fig. 3a-f) the tendency of macrophages, while in Hsp70-mediated fusion receptor expression in TAMs, it was founded that TAMs treated with Hsp70 resulted in significant increase in the expression of CD80 (53.23±12.3)and CD86 receptors (69.4±15.34)as compared to the TAMs of CD80 (13.23±2.3) and CD86 (19.4±5.34) incubated in medium alone, which is corresponding to ($p < 0.001$) the observation that hsp70 treatment resulted in increase in the tendency of TAMs.

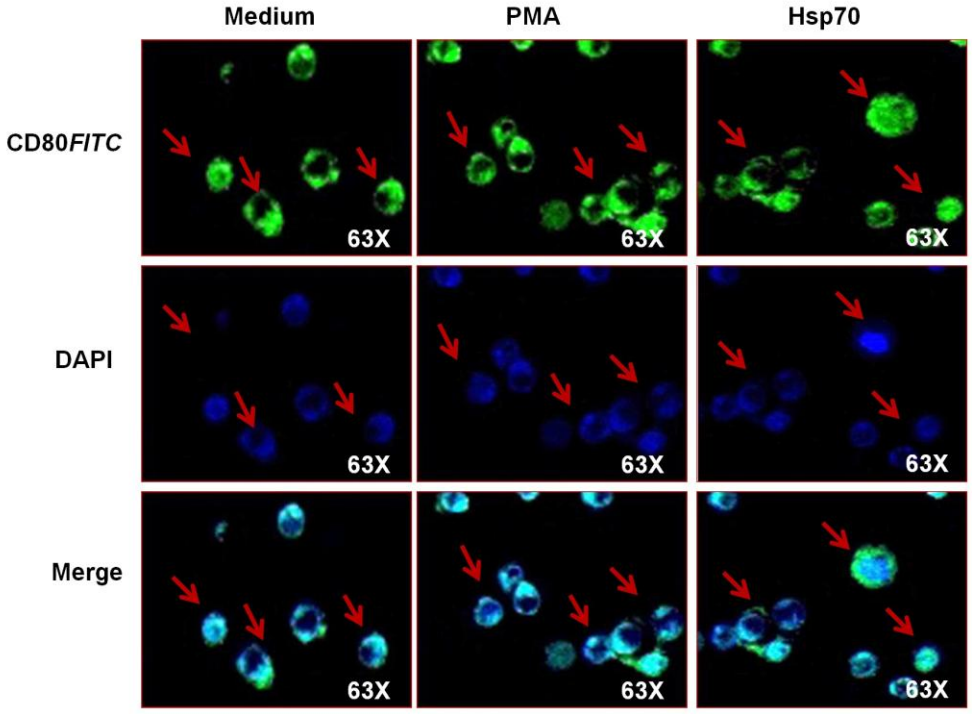

Fig. 3(a) (CD80 NMO)

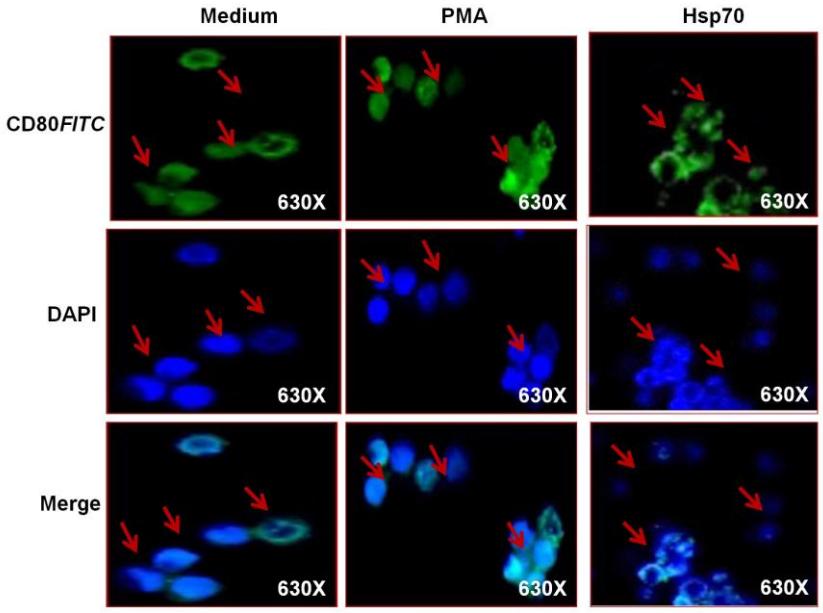

Fig. 3(b) (CD80 TAMs)

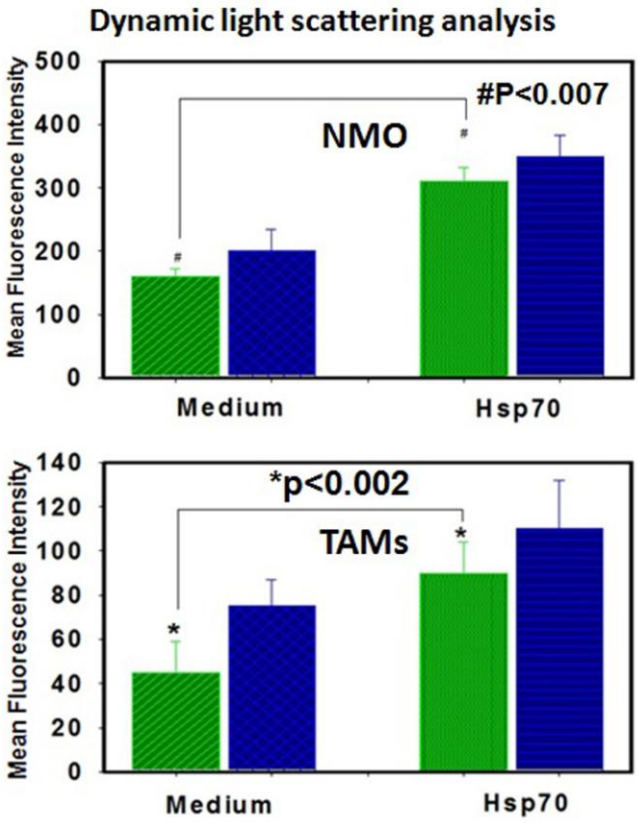

Fig. 3(c)

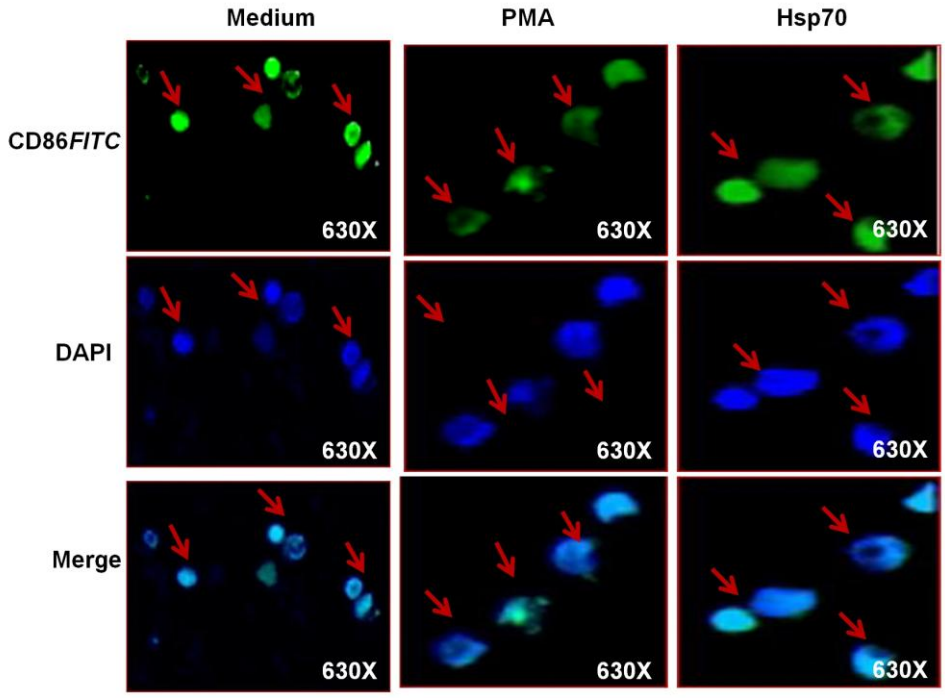

Fig. 3(d) (CD86 NMO)

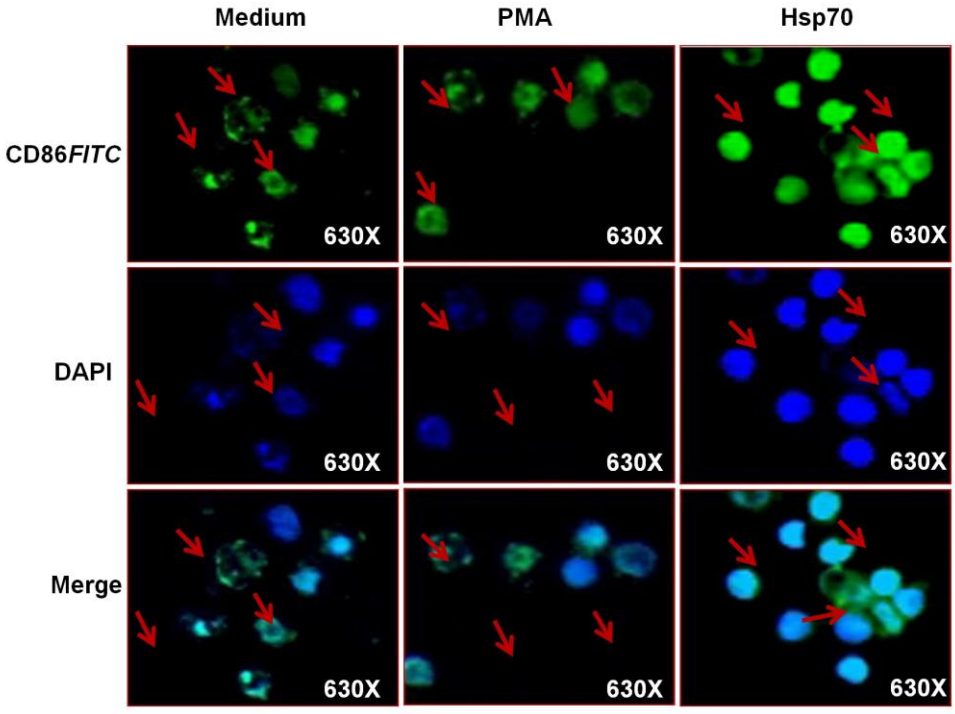

Fig. 3 (e) (CD86 TAMs)

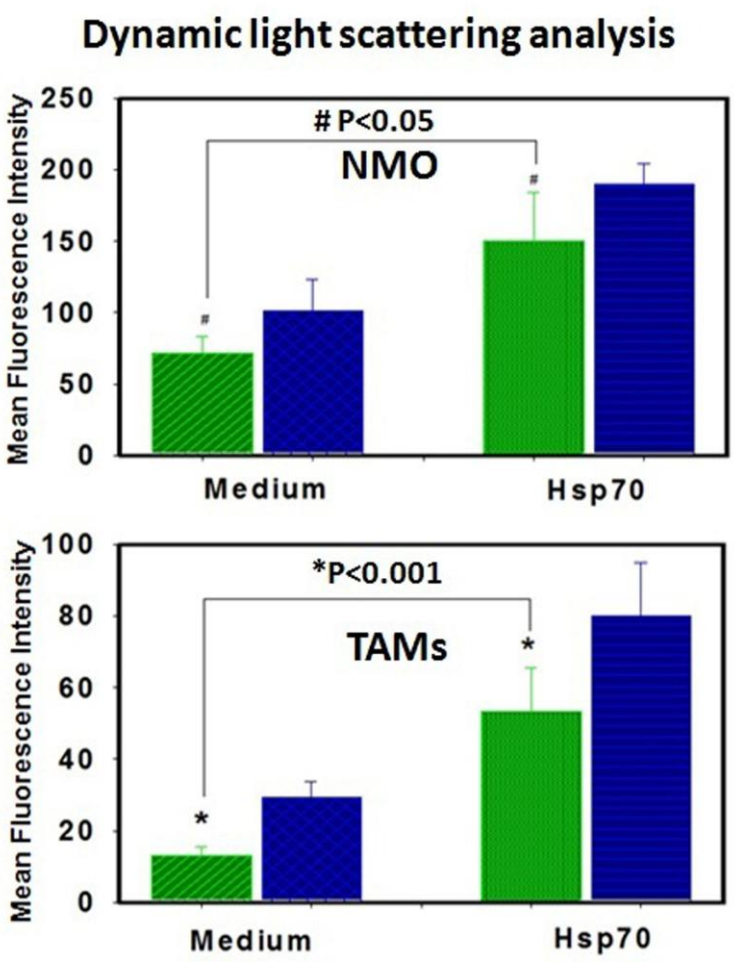

Fig. 3 (f)

Flowcytometric result shows autologous Hsp70 enhances the expression of CD80/CD86 molecules on NMO and TAMs

The expression level of CD80/CD86 molecules on the surface of NMO and TAMs was studied with the help of flowcytometry. NMO and TAMs harvested from different group of mice were incubated in medium and medium with or without PMA and Hsp70 and expression of co-stimulatory receptor molecules (CD80 and CD86) were observed by flowcytometry. It was found that NMO treated with Hsp70 resulted in significant increase in the expression of CD80 (140± 13.21) and CD86 (135±12.13) receptor as compared to the expression of CD80 (68.34±16.90) and CD86 (58±10.03) on NMO incubated in medium only (Fig.4a, 4b). Similarly, TAMs treated with autologous Hsp70 showed significant increase in the expression of CD80 (51± 16.01) and CD86 (63±9.13) receptor as compared to the expression of CD80 (9.09±5.90) and CD86 (11.62±7.03) on TAMs incubated in medium only.

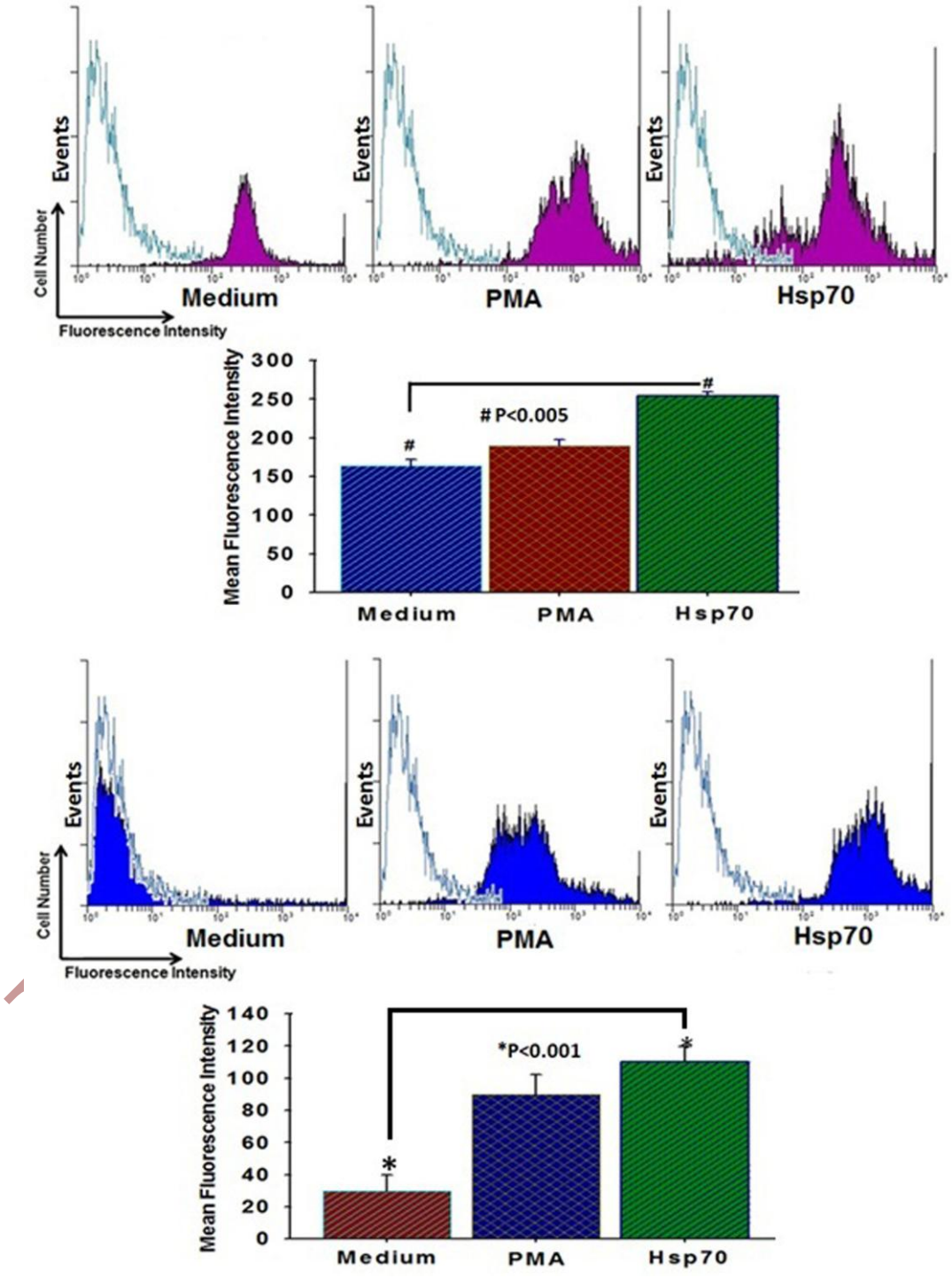
Events
Cell Number
Fluorescence Intensity
Medium
PMA
Hsp70
Mean Fluorescence Intensity
300
250
200
150
100
50
0
# P<0.005
#
*P<0.001
*
140
120
100
80
60
40
20

Fig. 4 (a)

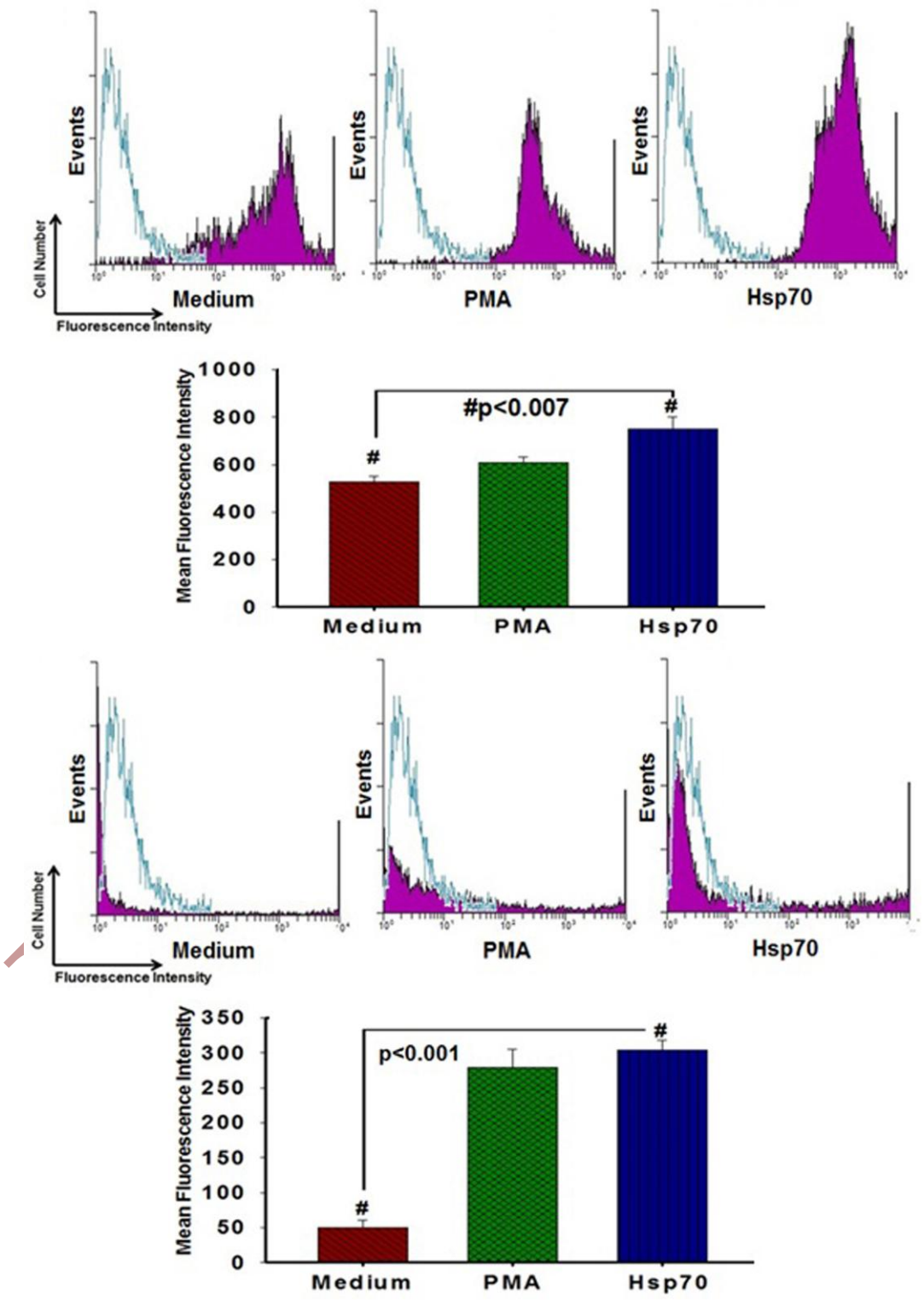
Events
Cell Number
Fluorescence Intensity
Medium
PMA
Hsp70
Mean Fluorescence Intensity
1000
800
600
400
200
0
#p<0.007
#
Medium
PMA
Hsp70
Events
Cell Number
Fluorescence Intensity
Medium
PMA
Hsp70
Mean Fluorescence Intensity
350
300
250
200
150
100
50
0
p<0.001
#
Medium
PMA
Hsp70

Fig. 4 (b)

Immunoblotting result shows autologous Hsp70 enhances the expression of CD80/CD86 molecules on NMO and TAMs

Western blot analysis of co-stimulatory receptor CD80/CD86expression in cells shows a band with an estimated molecular weight of<44-60kDa, 75-115kDa (known molecular mass of protein) respectively. It was found that NMO treated with Hsp70 resulted in significant increase in the expression of CD80 (66%) and CD86 (77.7%) receptor as compared to NMO incubated in medium only (Control) (Fig.5a-c). Similarly, TAMs treated with Hsp70 resulted in significant increase in the expression of CD80 (128%) and CD86 (156%) receptor as compared to the TAMs incubated in medium alone, which is corresponding to $p<0.001$.

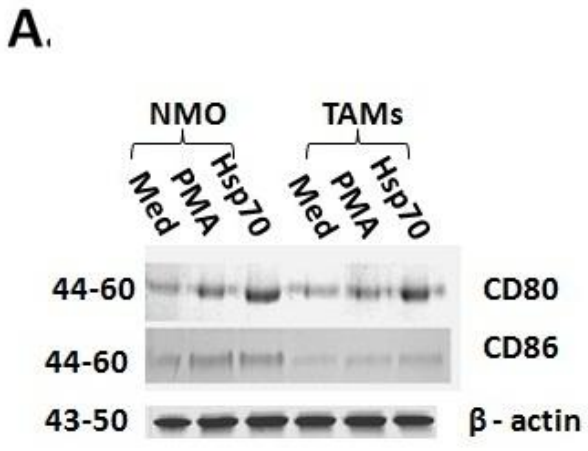

Fig. 5 (a)

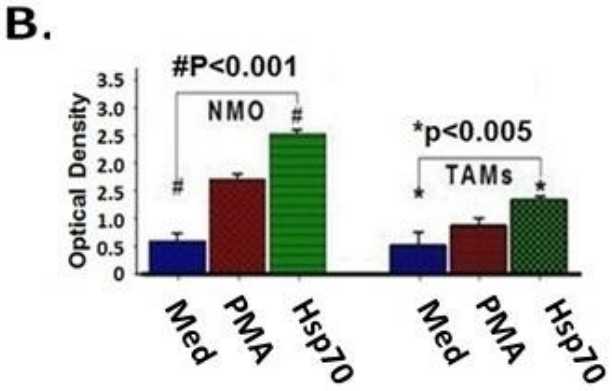

Fig. 5 (b)

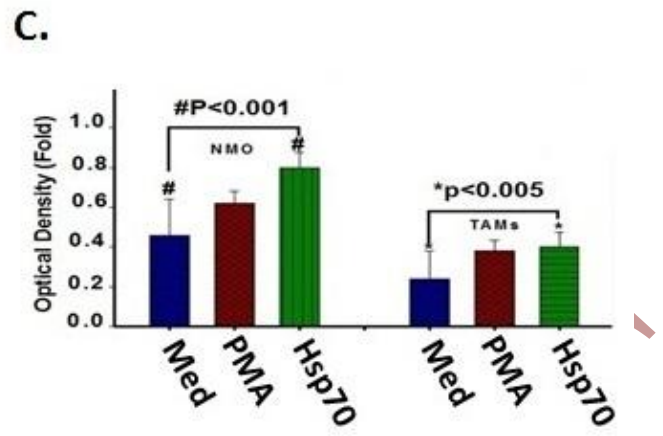

Fig. 5 (c)

Effect of autologous Hsp70 on mRNA expression of CD80/CD86 in NMO and TAMs

Experiments were conducted to examining the effect of hsp70 mediated co-stimulatory receptor expression in NMO and TAMs harvested from different group of mice, cells were incubated in medium and with or without PMA and Hsp70 and expression of CD80 and CD86 mRNA was observed. It was found that NMO treated with Hsp70 showed significantly increased expression level of mRNA ofCD80 (55%) and CD86 (27.4%) receptor as compared to the expression level of mRNA of CD80 and CD86 molecules incubated in medium only (Control), which is corresponding to $p<0.05$ (Fig.6a-c). Similarly, TAMs treated with Hsp70 showed increased expression of mRNA of CD80 (187%) and CD86 (176%) receptor as compared to the expression level of mRNA of CD80 and CD86 molecules incubated in medium only (Control) which is corresponding to $p<0.001$.

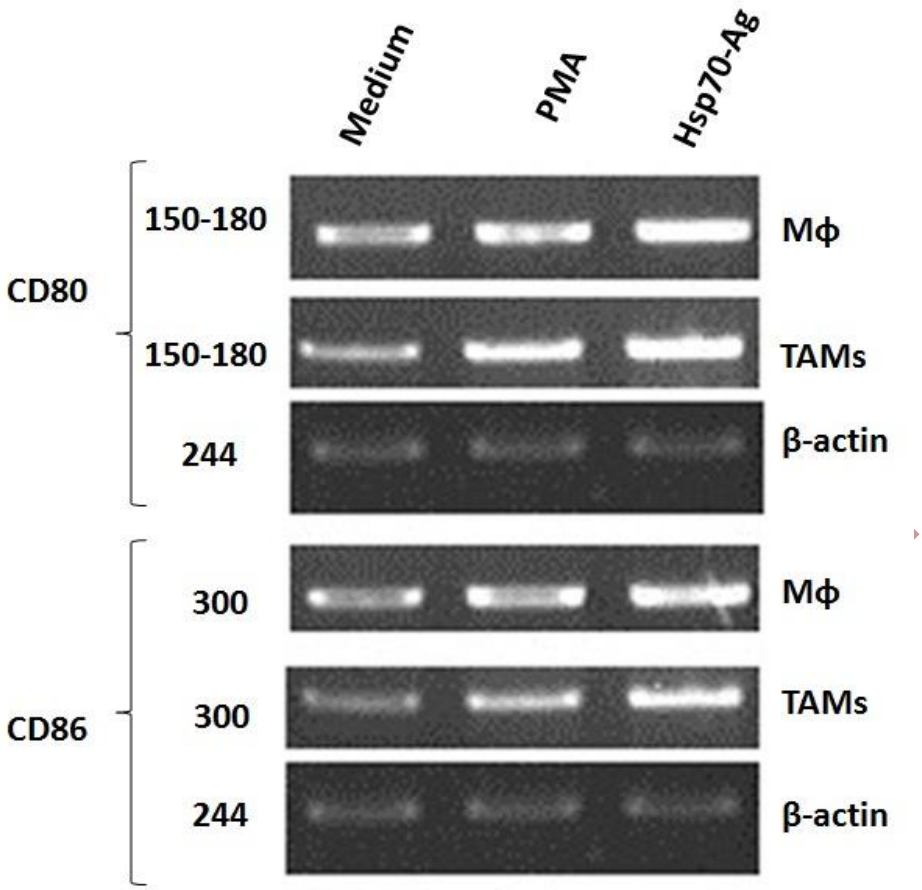
Medium
PMA
Hsp70-Ag
CD80
150-180
Mϕ
150-180
TAMs
244
β-actin
CD86
300
Mϕ
300
TAMs
244
β-actin

Fig. 6 (a)

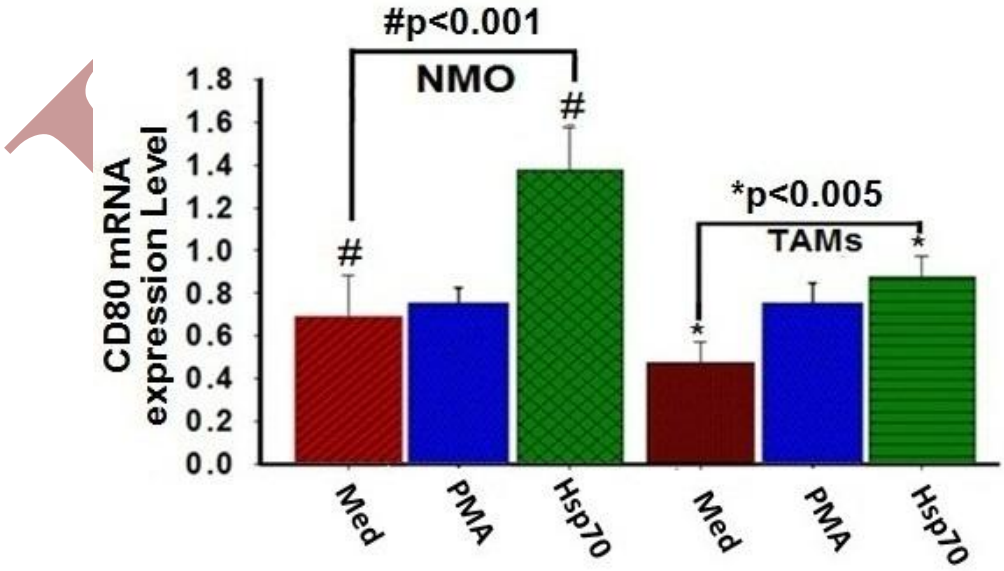
#p<0.001
NMO
*p<0.005
TAMs
CD80 mRNA expression Level
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Med
PMA
Hsp70
Med
PMA
Hsp70

Fig. 6 (b)

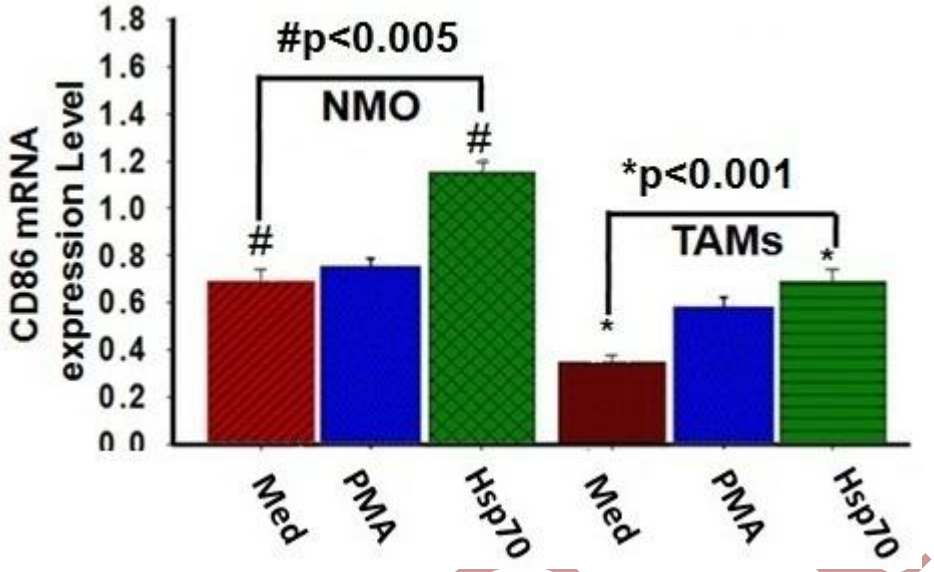

Fig. 6 (c)

DISCUSSION

In the present study, autologous Hsp70-tumor associated peptide complex (Hsp70) purified from Dalton's lymphoma cells and determined its effect on tumor promoting TAMs harvested from DL bearing mice to study the expression of co-stimulatory molecule CD80 and CD86. Resulting data demonstrated the (i) expression level of CD80 and CD86 in MØs and TAMs and (ii) or its increase expression upon autologous Hsp70-peptide treated MØs and TAMs; suggesting the direct effect of autologous Hsp70-peptide on MØs and TAMs. The expression of B7 molecules in malignancies is unclear. Some studies demonstrating elevated CD80 expression 3 days after cold I/R injury and reduced CD86 expression on monocytes derived from patients with septic shock (26- 28). Some work has been reported from our lab and other workers that suppressed function of alternatively activated macrophages has been retained by the treatment of 10µg/ml of autologous hsp70 and led to enhanced production of various Th1 and Th2 cytokines, chemokines and reactive intermediates metabolites such as RNI and ROI (29, 30), increased number of narrow, elongated lamellipodia and enhanced adherence as compared to macrophages, enhanced anti-tumor cytokine production multinucleation, MFR expression (CD172α and CD47) and enhanced MHC expression reported from our lab (31-32).

Despite the assets of signals that emanate from the TCR in the absence of co-stimulation, TCR signaling alone only leads to a brief proliferative burst and subsequent induction of an unresponsive state known as anergy or tolerance. Co-stimulatory signals can be delivered by a variety of signaling receptors of APCs (28). Co-stimulatory signals are required for the formation of the immunological synapse, a supramolecular complex and pre-requisite for sustained TCR signaling. Sustained TCR signaling is itself a prerequisite for full T cell activation (29). CD80

and CD86 play important role in the activation of T cell stimulation, CD80 favor the Th1 cell function while CD86promte the activation of Th2 responses on APCs (36, 37).

It is well known and documented that B7.1 (CD80)-binding stimulates a Th1 response, promote the secretion of cytokines such as e.g., IL-2, interferon-γ, that promote cellular immune responses. It was been found that the expression of CD80 was drastically decreases in TAMs (13.23±2.3) harvested form tumor bearing mice as compare normal phenotype of macrophages (71.3± 12.2) harvested from normal mice. Impact of the decreased release of Th1 cytokines might be promote tumor progression (9, 29).

B7.2 (CD86) stimulate Th2 response, which is characterized by anti-inflammatory cytokines (e.g. IL-4, IL-10, TGF-β) that promote development of immune cells. It was found that the expression of CD86 was decreases in TAMs (19.4±5.34) harvested form tumor bearing mice as compare normal phenotype of macrophages (53±9.23) harvested from normal mice(19, 29).

The decreased expression of CD80/CD86 facilitate the tumor progression by loss of co-stimulation, cell to cell contact (APC-B 7: T cell-CD28/CTLA-4) results T cell anergy (19) instead of activation and drastically affect the production of Th1 and Th2 cytokine, as a result skewing of immune responses against tumor regression. It has been reported that, during tumor progression, phenotype and functions of TAMs regulated by cytokines, tumor derived factor and local intracellular microenvironment which transform M1 phenotype of MØs into polarized tumor associated M2 phenotype (TAMs). These TAMs are further responsible for suppressed adaptive anti-tumor immune responses. TAMs express vast range of immune factors such as IL-4, IL-10, for M2 growth and differentiation, IL-1, 6, 8, bFGF, CSF, TGF-β for angiogenesis, IL-23 invasion and MMPs for metastasis of tumor cells Cell–cell fusion might be a novel repair mechanism whereby MØs exploit their fusion ability to revive damaged somatic cells. There is also increasing evidence that MØs fused with tumor cells to trigger the metastatic process while this possibility remains enigmatic, it makes sense if one considers that two important characteristics of MØs are motility and adaptation to new microenvironments (29, 30).

On the other hand, basal level of CD80 and CD86 expression was detected in TAMs harvested from tumor bearing mice was enhances upon TAMs incubation with Hsp70-peptide complex (53.23±12.3 for CD80, 69.4±15.34 for CD86) but it was at lesser extent as compare to MØs upon incubation with hsp70-peptide (150±34 for CD80, 198± 45.1 for CD86). Data shows that hsp70 significantly enhances the suppressed expression of CD80 and CD86 in TAMs results hsp70- peptide complex revert the suppressed property of TAMs (M2) to Slightly M1 activated phase which act against tumor progression by releasing various Th1 and Th2 anti-tumor cytokines that remarkably enhances the other immune cell function that suppress the tumor progression (26).

In conclusion, we document a pivotal role for Hsp70-peptide complex interaction in regulating the Costimulatory receptor CD80/86 expression during tumor progression. These data suggest

that any future therapies targeting these molecules in malignancies be directed specifically at CD80/CD86 expression.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENT

We are thankful to Dr. S.S. Agarwal, IMS, BHU, varanasi for providing flowcytometry facility for the analysis of receptors, ISLS, BHU, for confocal microscopy Imaging.

REFERENCES

1. Forngaleg-Behia EB, Doseff AI. Regulation of monocytes and macrophages cell fate. *Front Bios* 2008;14:2413-31.
2. Lewis CE, Pollard JW. Distinct Role of Macrophages in Different Tumor Microenvironments. *Can Res* 2006;66(2):605-12.
3. Subauste CS, Malefyt RDW, Fuh F. Role of CD80 (B7.1) and CD86 (B7.2) in the Immune Response to an Intracellular Pathogen. *J Immunol* 1998;160:1831-40.
4. Costello RGT, Mallet FO, Sainty D, et al. Regulation of CD80/B7-1 and CD86/B7-2 molecule expression in human primary acute myeloid leukemia and their role in allogenic immune recognition. *Eur J Immunol* 1998;28:90–03
5. Azuma M, Ito D, Yagita H, Okumura K, Phillips JH, Lanier LL, Somoza C. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* 1993;366:76-79.
6. Freeman GJ, Gribben JG, Boussiotis VA, Ng JW, Restivo VA Jr, Lombard LA, Gray GS, Nadler LM. Cloning of B7-2: a CTLA-4 counter- receptor that costimulates human T cell proliferation. *Science* 1993;262:909-11.
7. Freeman GJ, Freedman AS, Segil JM, Lee G, Whitman JF, Nadler LM. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J Immunol* 1989;143(8):2714-22.
8. Olive D, Pages F, Klasen S, Battifora M, Costello R, Nunes J, Truneh A, Ragueneau M et. al. Stimulation via the CD28 molecule: Regulation of signaling, cytokine production and cytokine receptor expression. *Fundam Clin Immunol* 1994;2:185–97.
9. June CH, Bluestone JA, Nadler LM, and Thompson CB. The B7 and CD28 receptor families. *Immunol Today* 1994;15:321–31.
10. Schultze J, Nadler LM, and Gribben JG. B7- mediated costimulation and the immune response. *Blood Rev* 1996;10:111–27.

11. Guinan EC, Gribben JG, Boussiotis VA, Freeman GJ, and Nadler LM. Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. *Blood* 1994;84: 3261–82.
12. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 1996;14:233-58.
13. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28- mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 1992; 356:607-09.
14. Tamura H, Dan K, Tamada K, Nakamura K, Shioi Y, Hyodo Hideya. Expression of Functional B7-H2 and B7.2 Costimulatory Molecules and Their Prognostic Implications in De novo Acute Myeloid Leukemia. *Clin Can Res* 2005;11:5708-17
15. Mantovani A, Sozzani S, Locati M, Allavena P and Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trend Immunol* 2002;11:549-55.
16. Bingle L, Brown NJ, Lewis CE. The role of tumor-associated macrophages in tumor progression: implications for new anticancer therapies. *J Pathol* 2002;3:254-65.
17. Sarireh BL and. Eremin O. Tumor-associated macrophages (TAMS): disordered function, immune suppression and progressive tumor growth. *J R Coll Surg* 2000;45:1-16
18. Mahoney KH, Heppner GH. Facs analysis of tumor- associated macrophages replication: differentiation between metastasis and nonmetastasic murine mammary tumor. *J leuko Boil* 1987;41:205-11.
19. Solinas G, Germano G, Mantovani A, and Allavena AP, et al. Tumor-associated macrophages (TAM) as major players of the cancer-related Inflammation. *J leuko boil* 2009; 86:1065-73.
20. Siveen KS and Kuttan G. Role of macrophage in tumor progression. *Immunol lett* 2009; 123:97-02.
21. Jattela M. Heat shock proteins as cellular lifeguards. *Ann Med* 1999;31:261-71.
22. Tamura Y, Peng P, Liu K et al. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* 1997;278:117-20.
23. Udono H, Srivastava PK. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* 1993;178:1391-96.
24. Tokuda Y, Tsuji M, Yamazaki M et al. Augmentation of murine tumor necrosis factor production by amphotericin B in vitro and in vivo. *Antimicrob Agents Chemother* 1993; 37:2228-30.
25. Blachere NE, Li Z, Chandawarkar RY, Suto R, Jaikaria S, Basu S, et al. Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytoxic T-lymphocytes response and tumor immunity. *J Exp Med* 1997;186:1315-27.

26. Peng P, Menoret A and Srivastava PK. Purification of immunogenic heat shock protein 70-peptide complexes by ADP-affinity chromatography. *J Immunol Meth* 1997; 204:13-21.
27. Shahoo NC, Rao KVS, Natrajan K. CD80 expression is induced on activated B cell following Stimulation by CD86. Scnd J immunol 2002;55:577:84.
28. Lahat N, Rahat MA, Ballan M, Weiss-Cerem L, Engelmayer M, Bitterman H. Hypoxia reduces CD80 expression on monocytes but enhances their LPS-stimulated TNF-α secretion. J Leukoc Biol 2003;4(2):197-5.
29. Friedemann K, Wolfgang FV, Ruediger A. Signal transduction and co-stimulatory pathways. Transp Immunol 2002;9:69–82.
30. Kuchroo VK, Das MP, Brown JA, Ranger AM., Zamvil SS, Sobel RA, Weiner HL, Nabavi N, Glimcher LH. B7–1 and B7–2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell 1995; 80:707–18.
31. Harris NL, Prout M, Peach RJ, Fazekas DSG, B Ronchese, F. CD80 costimulation is required for Th2 cell cytokine production but not for antigen-specific accumulation and migration into the lung. *J. Immunol* 2001;166:4908–14.
32. Anderson DE, Sharpe AH, Hafler DA. The B7-CD28/ CTLA-4 costimulatory pathways in autoimmune disease of the central nervous system. Curr Opin Immunol 1999;11:677–83.
33. Kumar S, Deepak P, Acharya A. Hsp70 induces Th1 polarization through tumor-associated macrophages in a T-cell lymphoma. Neoplasma 2007;54(2):113–22.
34. Gautam PK, Kumar S, Deepak P, Acharya A. Morphological effects of autologous hsp70 on peritoneal macrophages in a murine T cell lymphoma. Tumor Biol 2013;34:3407–15.
35. Simone M, Clara B, Pierluigi P. Coinhibitory molecules in cancer biology and therapy. Cyto& Grow Fact Revi 2013;24:147–61.
36. Lin RY, Saha DC, Bernstein CJ, Astiz ME, Rackow EC. The pattern of inflammation in rat sepsis due to enterotoxin-producing staphylococcus aureus: a comparison with ischemia-reperfusion injury. J Med 1996; 25:303–17.
37. Takada M, Chandrakerm A, Nadeaum KC, Sayeghm MH, Tilneym NL. The role of the B7 co-stimulatory pathway in experimental cold ischemia/reperfusion injury. J Clin Invest 1997;100:1199–03.