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Autologous Hsp70 immunization induces anti-tumor immunity and increases longevity and survival of tumor-bearing mice

S. KUMAR, P. DEEPAK, A. ACHARYA*

Immunology Laboratory, CAS in Zoology, Banaras Hindu University, Varanasi – 221 005, India. E-mail – acharya@bhu.ac.in.

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Heat Shock proteins 70 (Hsp70) is a family of highly conserved molecules that maintain the function of crucial cellular pathways during stress. Hsp70 derived from tumor cells is bound with tumor antigenic peptides from the diverse antigen cytosolic pool. Tumor-derived Hsp70 preparations after *ex vivo* administration permit antigen presenting cells (APCs) to present tumor antigen to their cell surface and induce tumor specific immunity in many types of malignancies by directly eliciting cytotoxic T lymphocytes (CTL) response. In the present investigation, we have demonstrated that immunization with tumor cell derived Hsp70 lead to an effective survival advantage in mice with minimal residual tumor cells from which Hsp70 is derived, by involvement of immune cell types in the rejection of tumor in the Hsp70 immunized tumor-bearing host mice and their post-immunization cytokine repertoire.

It has been observed that autologous Hsp70 induces specific anti-tumor immunity and effectively eradicates tumors in the host mice, thereby enhancing survival of tumor-bearing host. Immunization with tumor-derived autologous Hsp70 effectively primed specific CTL response and increased tumor cell lysis independently of $CD4^+$ T lymphocytes. Increase in type I cytokines in the serum of Hsp70 immunized mice was also observed that indicates its adjuvant property in the host. Furthermore, Hsp70 immunized mice did not show any systemic disorder. Therefore, it could be assumed as safe and might be clinically useful for vaccination against malignant human tumors.

Key words: Hsp70, Immunization, Survival, CTL response, Pro-inflammatory cytokines

Heat Shock proteins 70 (Hsp70) are a family of highly conserved molecules with a relative molecular mass of 70 kD. They are present in all living cells and in most compartments of eukaryotic cells. They can exist in an unbound state or bound to specific client proteins and function as molecular chaperons in numerous processes, such as protein folding, assembly and transport, peptide trafficking, antigen processing under physiologic and stress conditions [1–8]. Expression of Hsp70 can be induced by several stressors such as fever, oxidative stress, alcohol, inflammation and heavy metals, and by conditions causing injury and necrosis, such as infection, trauma and ischemic reperfusion. They serve to attenuate the damage and misfolding of proteins caused by these various stressors. HSPs bind to exposed hydrophobic sites on polypeptides and mediate conformational changes, prevent misfolding of peptides and facilitate transport across membranes, thus maintaining the function of crucial cellular pathways during stress.

Hsp70 has been demonstrated to stimulate tumor specific immunity in many types of malignancies, since the first identification of anti-tumor activity of HSPs, gp96–peptide complexes in a rat model [9] and later on in a murine model [10, 11]. The immunogenicity of Hsp70 preparations from tumor cells has been attributed to peptides bound to Hsp70 proteins by virtue of its protein folding [12]. Autologous Hsp70 from tumor cells are able to induce cross priming of T cells [13], which suggest that HSP proteins potentially chaperones tumor antigenic peptides, process and load them onto MHC class I molecules, where they can be presented to cytotoxic $CD8^+$ T cells CTL response [14–20]. It is due to this reason, tumor-derived HSP-antigenic peptide complexes have been purified for immunotherapy vaccination against cancer [21] without the need to identify antigenic tumor peptides associated with them [22]. Hsp70 purified from cells are associated with a broad range of peptides from diverse antigenic reper-

* Corresponding author

toire of the tumor cells from which they are purified [14]. Besides the potential role of Hsp70 to elicit $CD8^+$ CTL responses, it has been known to stimulate macrophages to elaborate cytokines and induce expression of higher levels of co- stimulatory molecules on the dendritic cells [23–25] through the involvement of the NF_kB pathway [26]. We have also previously demonstrated that autologous Hsp70 are able to induce Th1 polarization by activating macrophages in a murine T cell lymphoma [27]. This Th1 polarizing property of Hsp70 can be taken as reminiscent of macrophage stimulatory property of lipopolysaccharides and interferon-γ. It has been argued that a balance of Th1 and Th2 type of cytokine is critical to the outcome and maintenance of inflammatory and immune response. Tumor progression results into an imbalance of cytokine at the milieu [28, 29]; with the Th1 cytokine providing the cellular immunity necessary for anti-tumor immune response whereas Th2 type of cytokines dampens the cellular immunity in the tumor-bearing host.

Owing to the property of autologous Hsp70 to induce anti-tumor cytotoxic $CD8^+$ T cells CTL response and adjuvanticity in the tumor-bearing host, we sought to investigate the changes in post-immunization cytokine milieu in the tumor-bearing host and the effect of exogenous application of autologous Hsp70 on the survival of murine host bearing a spontaneously transplantable T cell lymphoma designated as Dalton's Lymphoma and Sarcoma.

Materials and methods

Experimental animals. Inbred populations of BALB/c ($H\text{-}2^d$) strain of mice of either sex at 6–8 weeks of age were used in this investigation. 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, Banaras Hindu University.

Cell culture and Hsp70 induction. The murine T- cell lymphoma cell line, DL (Dalton's lymphoma) and murine sarcoma cell line (S-180) were obtained from National Centre for Cell Science (NCCS), Pune, India, and were maintained in ascitic form by serial transplantation to the healthy mice. Alternatively, cells were kept cryopreserved for future reference. Tumor cells were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 0.5% 2-mercaptoethanol (ME) at 37°C on 5% CO_2.

For HSP induction, heat shock treatment to both of the tumor cell culture was done. Cells were harvested in 1mM EDTA in PBS, transferred to 12ml polypropylene tubes (Tarson, Kolkata, India), washed in HEPES buffered RPMI 1640 medium, and resuspended in this medium containing 10% FBS. Cells were exposed to 42° C for 1 hour in a water bath or kept at 37°C as controls. After a recovery period of 4 hours at 37°C, the cells were used for additional experiments.

Purification of Hsp70. The Hsp70 were isolated and purified as previously described [18] with minor modifications. A total of 10ml packed all pellets 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) loaded onto Sephadex column (G-75 from Sigma). Further, elute from Sephadex column 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 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 the elute was loaded onto a DEAE-Sephacel column (Sigma) in buffer D (130mM NaCl in buffer A). The protein content of different fractions was measured by the Lowry method with BSA as standard.

To isolate Hsp70 from Sarcoma Cell line, culture of S–180 cells in Petri dishes were washed with PBS containing 0.05% Trypsin/EDTA solution for 10 minutes to prepare single cell suspensions. Suspension was centrifuged at 300 x g for 5 min, resuspended in lysis buffer and Hsp70 was isolated by the same method as applied for DL cells.

Immunotherapy with Hsp70. 3×10^6 DL cells were transplanted by intra–peritoneal injection and same number of S–180 cells was transplanted subcutaneously to the flank region of healthy mice of 6 to 8 weeks of age. Day of Transplantation was appointed as day 0. After tumor cells transplantations, 50μg of hsp70 derived from DL cells were injected intravenously into tail vein of the DL bearing mice on days 4, 9, 14 and 19. Similarly, 50 μg of Hsp 70 derived from S–180 cells were injected intravenously into tail vein of the mice bearing S–180 tumor on days 4, 9, 14 and 19. Control group of mice were treated with only sterile PBS (137mM Nacl, 8.1 mM $Na_2HPO_4.12H_2O$, 2.68 mM KCl, 1.47mM KH_2PO_4). Survival times of the autologous-Hsp70 immunized mice after DL cells and S–180 transplantation were composed using the Kaplan and Meier method. For the experiments to determine the specificity of Hsp70 derived from DL cells and S–180 cells, 50μg of Hsp70 derived from liver of healthy mice was injected intravenously into tail vein.

In vivo depletion of $CD4^+$ or $CD8^+$ T cells from mice was carried out during the immunotherapy with Hsp70 as described previously [17, 22]. Briefly, 200ul of 1:8 diluted anti- $CD4^+$ (clone GK 1.5) or anti-$CD8^+$ (clone 2.43) antibodies were intravenously injected on days 2, 7, 12 and 17, each time 2 days before immunization of Hsp70. Depletion efficacy is known to be between 90% to 100% by this method [30].

Estimation of tumor cell survival in vitro by MTT assay. Mixed lymphocyte-tumor cell culture was grown *in vitro* as previously described [31]. Briefly, 1.0×10^3 splenocytes of Hsp70 vaccinated mice were cultured for 5 days with 1.0×10^5 irradiated DL cells and S–180 cells in a 1 ml volume in RPMI 1640 culture medium containing 10% FCS and 0.5%

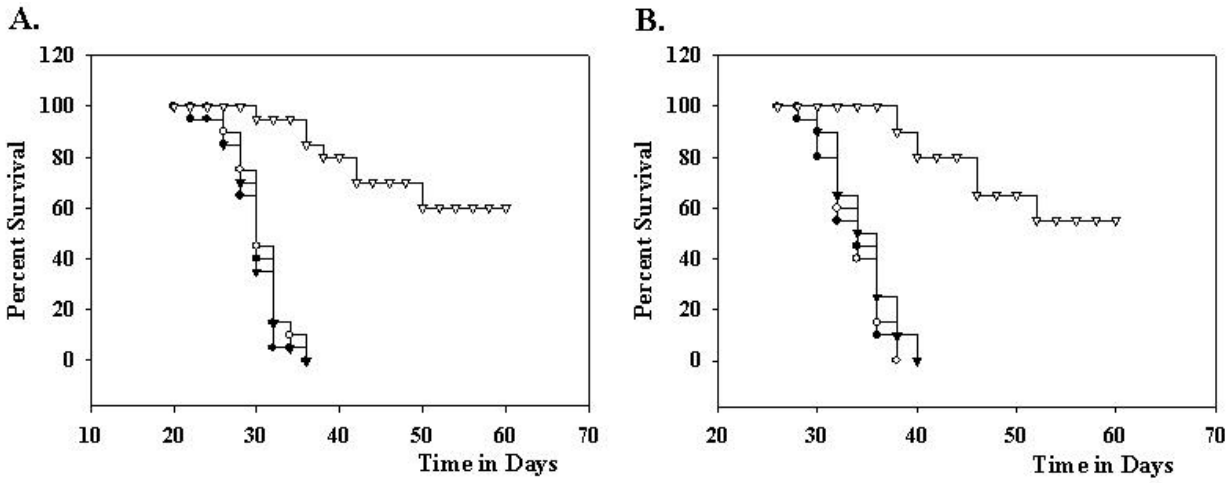

Fig. 1. Percent survival of tumor-bearing mice. The mice were transplanted with 3 x 10^6 DL-cells by intraperitoneal injection and 3 x 10^6 S-180 cells were transplanted by intradermal injection in 0.5 ml of PBS. The mice were injected intraperitoneally with sterile PBS (—●— , n = 20), autologous Hsp70 (—▽—, n = 20), Hsp70 derived from healthy liver (—○—, n = 20) and Hsp70 derived from syngenic tumor (—▼—, n = 20) on days 4, 9, 14 and 19 as described in Materials and methods and survival analysis was done. The survival of the mice was observed and potted on Kaplan–Meier plot. P < 0.005 (log-rank test). A. Show the percent survival of DL-bearing host and B. show the percent survival of mice bearing sarcoma (S-180).

mercaptoethanol, 200µl of this suspension was seeded in each well of round bottom plate then MTT assay was carried out as described previously [32, 33]. After incubation of mixed lymphocyte-tumor cell culture, cells were incubated for 4 hrs with 50µl of 0.8 mg/ml of MTT at 37°C. The plate was then centrifuged at 100 x g for 5 min at 4°C (Remi, New Delhi, India). The supernatant was then carefully removed without disturbing the dark blue formazan crystals. 100 µl of the DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals. The absorbance was then read on a micro plate reader (Bio-Rad, CA, USA) at a wavelength of 540 nm. The reading was determined by subtracting the absorbance of culture containing only spleen cells as a control.

Serum preparation. For serum preparation, blood from both male and female adult DL– bearing and S–180 bearing mice were collected at different time interval by retro-orbital bleeding. The blood was kept at room temperature for 45-60 min and then after separating the clot from eppendorf wall, kept overnight at 4°C, Serum was then collected, centrifuged at 300 x g for 15 min to separate hemolysed cells. After centrifugation, serum was finally collected and stored at –20°C for the experiments.

Cytokine assay. Cytokine released into culture supernatants was determined by sandwich ELISA. Briefly, 96 well microtitre plate (Tarson, Kolkata, India) was coated overnight at 4°C with 50 µl of monoclonal antibodies against IL-2, IFN-γ, TNF-α, IL-10 and IL-13 diluted with coating buffer (0.1mM $NaHCO_3$, 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 200 µl of blocking buffer (PBS/ 2% BSA) for 1 hr at 37°C. The plates were then washed three times with washing buffer. Thereafter 50µl of standard IL-2, IFN-γ, TNF-α, IL-10 and IL-13, but same amount of culture supernatants was added in each well. The plates were then incubated for 2 hrs at 37°C. The plates were washed again three times and therefore, 100µl of goat IgG conjugated with alkaline phosphatase with dilution of 1:5000 were added in each well and incubated for 1 hr at 37°C. The plates were then extensively washed and incubated with p-nitro phenyl phosphate (1 mg/ml enzyme substrate buffer} for 45 min. 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.

Tumor regression assay. After immunotherapy, one mouse of each group was sacrificed for counting of DL cells or for histological examinations of the tumor, and remaining animals were observed for tumor growth. DL cells were simply counted on hemocytometer and calculated as number of DL cells per ml of ascitic fluid, whereas growth of S-180 was monitored by measuring diameter of the tumor mass. Tumor size of S-180 was followed in two perpendicular dimensions by using calipers and described as the tumor cross sectional area (mm^2).

Statistical method. All the experiments were done at least three times and data were taken as significant at $p<0.05$. The statistical analysis was performed by using two-tailed Student's t-test. The survival of mice was analyzed by log rank test and plotted on Kaplan-Meier plot. All statistical analysis was performed on statistical software package Sigma Plot Version 8.0 (Systat Software Inc., San Jose, CA, USA).

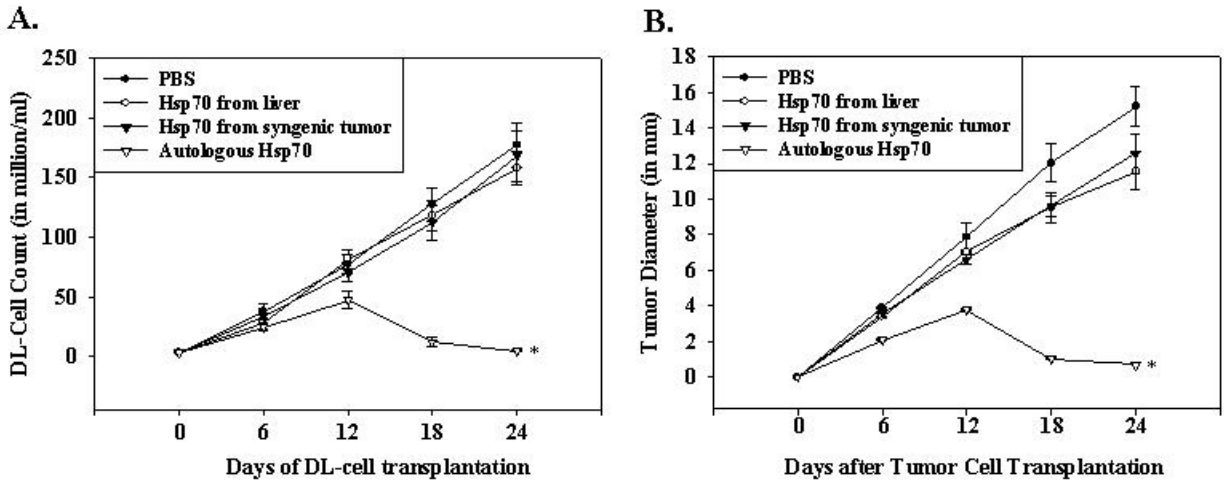

Fig. 2. Effect of immunization with autologous Hsp70 on DL cell count. The mice were transplanted with 3 x 10^6 DL cells by intraperitoneally injection and 3 x 10^6 S-180 cells were transplanted by intradermal injection in 0.5 ml of PBS. The mice were injected intraperitoneally with sterile PBS (—●— , n = 20), autologous Hsp70 (—▽—, n = 20), Hsp70 derived from healthy liver (—○—, n = 20) and Hsp70 derived from syngenic tumor (—▼—, n = 20) on days 4, 9, 14 and 19. The tumor cells were harvested at indicated time intervals and counted. The data are presented as mean DL-cell number/tumor diameter of at least three observations ± SE_M. The data are significant at P < 0. 005.

Results

Increase in longevity of mice injected with autologous Hsp70. Autologous Hsp70 preparations derived from DL cells were purified as described in "Materials and methods". The purified Hsp70 preparations were run on SDS-PAGE and bands were transferred to PVDF membrane (Immobilon, Millipore, USA) and reacted with anti-Hsp70 for confirmation (data not shown). These purified Hsp70 were injected intravenously into tumor-bearing mice at different time periods as described in Immunization with Hsp70 of the section Materials and methods. All the mice that received autologous Hsp70 (purified in adenosine 5'-diphosphate (ADP) column) survived for more than 40 days. In contrast, those mice received ADP column purified Hsp70 derived from normal liver and sterile PBS did not show any longevity and died within 32 days of DL cell transplantation (Fig. 1A). Further to test the efficacy of Hsp70, the mice were injected with Hsp70 derived from S-180 cells did not show any improvement in survival time and all DL-bearing mice died within 32 days of life span. These data suggested that repeated injection of autologous Hsp70 preparations derived from DL cells to DL-bearing mice resulted in both the rejection of DL cells and a survival enhancement (Fig. 2A).

The generality of the therapeutic efficacy of HSP70 immunization was then tested in other tumor of different etiology and histological origins, sarcoma generated by inoculating S-180 cell lines. Mice bearing the sarcoma were left untreated, treated with autologous Hsp70 or sterile PBS as a control after 4 days of tumor transplantation. Treatment with autologous Hsp70 resulted in a reduced growth rate of sarcoma in all mice (Fig. 1B). In this group of mice, tumor size on 24th days is as 48 days of post-tumor cell transplantation. When the mice were injected with sterile PBS, they did not show any reduction in growth rate of sarcoma in tumor-bearing mice (Fig. 2B). In contrast, the treatment with heterologous Hsp70 or Hsp70 derived from DL cells did not show any survival or longevity of tumor-bearing host.

Effect of $CD4^+$ and $CD8^+$ depletion in tumor-bearing host. To assess the efficacy of Hsp70 to induce cytotoxic T lymphocytes response and subsequent tumor cell elimination, we depleted mice of $CD4^+$ and/or $CD8^+$ T lymphocytes by anti-CD4 and anti-CD8 antibodies after tumor cell transplantation before the first dose of autologous Hsp70 and survival of mice were analysed by Kaplan-Meier method. Depletion of $CD8^+$ T lymphocytes completely abrogated the efficacy of Hsp70 immunization in both the tumor model, whereas $CD4^+$ T lymphocytes depletion also suppresses the growth of DL cells and S-180 in the host mice (Fig. 3 & 4). The mock depletion with a control IgG preparation had no effect in either of the tumor system. This observation is consistent with the previous observation [34], demonstrating that immunization with gp96 requires $CD4^+$ and $CD8^+$ T lymphocytes in inducing anti-tumor immunity. However, the requirement of NK cells in tumor eradication have also been proposed, this results indicates that both $CD4^+$ cells and $CD8^+$ T cells are required for eradication of tumor cells in the host mice immunized with autologous Hsp70.

Induction of CTL response against tumor cells in vitro. Following survival analysis of tumor bearing host mice, we next examined cytotoxic activities against DL cells and S-180 cells. Mice spleen cells from Hsp70 vaccinated and control mice were

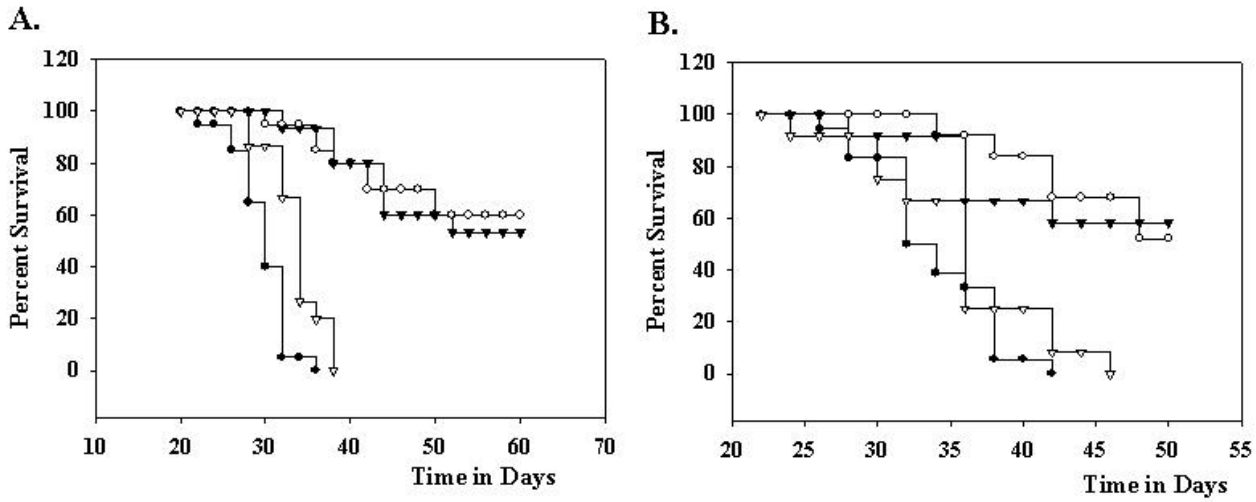

Fig. 3. Percent survival of tumor-bearing mice. The mice were transplanted with 3 x 10^6 DL-cells by intraperitoneally injection and 3 x 10^6 S-180 cells were transplanted by intradermal injection in 0.5 ml of PBS. The mice were injected intraperitoneally with sterile PBS (—●— , n = 20), autologous Hsp70 (—○—, n = 20), autologous Hsp70 + anti-CD4 antibody (—▼—, n = 15) and autologous Hsp70 + anti-CD8 antibody (—▽—, n = 15) on days 2, 7, 12 and 17, each time 2 days before immunization of Hsp70 as described in Materials and methods and survival analysis was done. The survival of the mice was observed and potted on Kaplan–Meier plot. P< 0.005 (log-rank test). A. Show the percent survival of DL-bearing host and B. show the percent survival of mice bearing sarcoma (S-180).

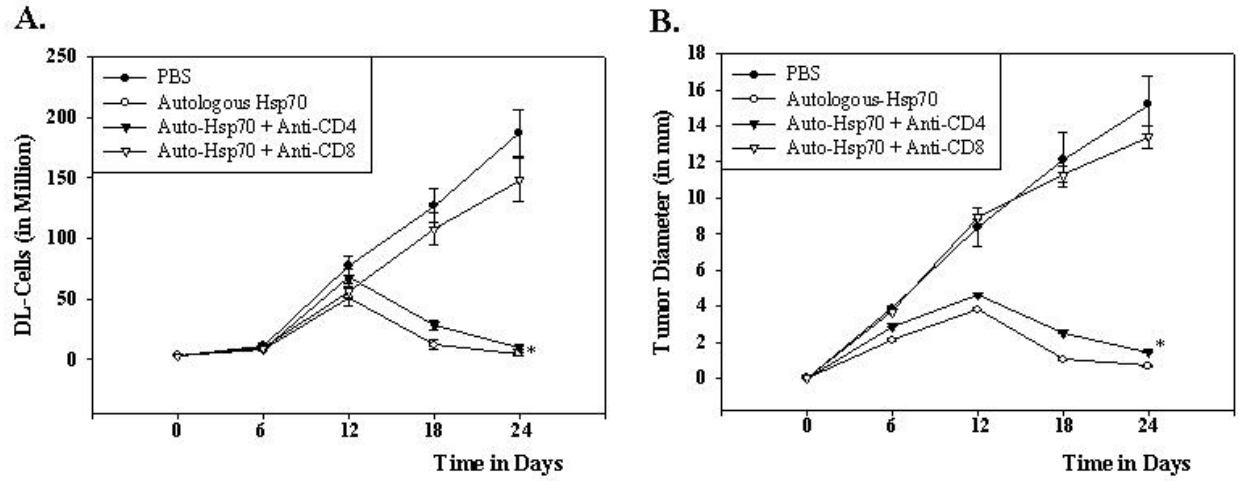

Fig. 4. Effect of immunization with autologous Hsp70 on DL cell count. The mice were transplanted with 3 x 10^6 DL cells by intraperitoneally injection and 3 x 10^6 S-180 cells were transplanted by intradermal injection in 0.5 ml of PBS. The mice were injected intraperitoneally with sterile PBS (—●— , n = 20), autologous Hsp70 (—○—, n = 20), autologous Hsp70 + anti-CD4 antibody (—▼—, n = 20) and autologous Hsp70 + anti-CD8 antibody (—▽—, n = 20) on days 2, 7, 12 and 17, each time 2 days before immunization of Hsp70. The tumor cells were harvested at indicated time intervals and counted. The data are presented as mean DL-cell number/tumor diameter of at least three observations ± SE_M. The data are significant at P< 0.05.

cultured with irradiated DL cells and S-180 cells and percent survival of tumor cells were determined by MTT assays as described in "Materials and methods". As shown in figure, spleen cells derived Hsp70 immunized mice showed significant cytotoxicities against corresponding DL cells (Fig. 5A) and S-180 cells (Fig. 6A) at the effector/target cell (E/T) ratio of 1:200, 1:100, 1:50. The spleen cells from control mice did not show significant cytotoxicities against corresponding tumor cells. This observation indicates that tumor rejection after Hsp70 immunization is due to induction in CTL response in these tumor systems. Further to determine the immune cells involved in tumor cell killing induced by Hsp70 immunization, spleen cells derived from Hsp70 immunized mice were pretreated with anti-mouse CD4 or anti-mouse CD8 before MTT assay was carried out. Anti-CD4 pretreatment did not block the cytotoxicity against DL cells (Fig. 5B) and S-180 cells (Fig. 6B), whereas

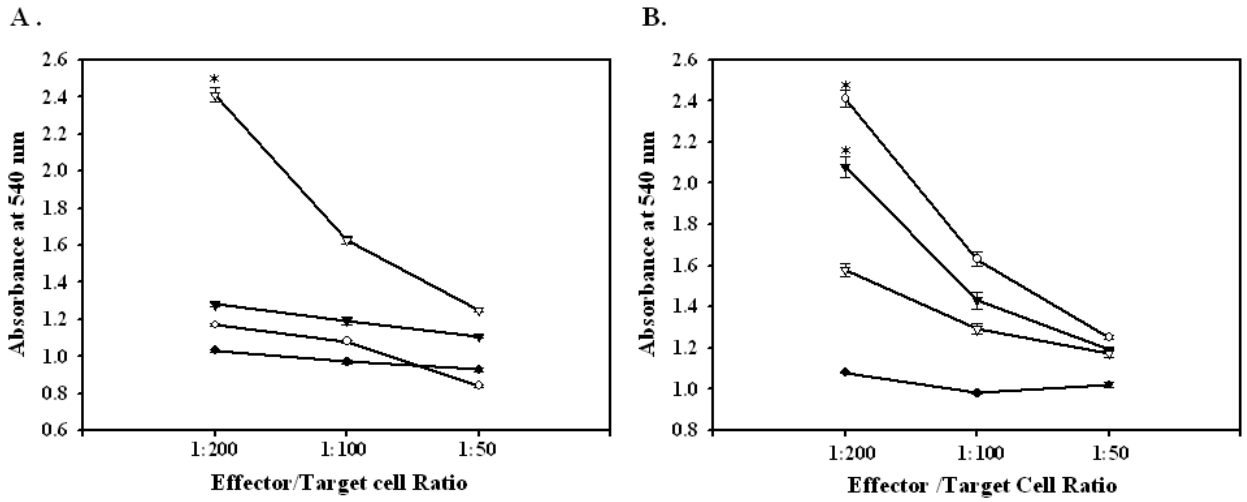

Fig. 5. Effect of autologous Hsp70 immunization and anti-CD4/CD8 treatment on cytotoxic activities of spleen cells against DL cells. After DL-cell transplantation, mice were injected *i.p.* with sterile PBS (—●—), 50 µg autologous Hsp70 (—▽—) or Hsp70 derived from healthy liver (—○—) or Hsp70 derived from syngenic tumor (—▼—) on days 4, 9, 14 and 19 (A.) and treated with sterile PBS (—●—), autologous Hsp70 (—○—), autologous Hsp70 + anti-CD4 antibody (—▼—) and autologous Hsp70 + anti-CD8 antibody (—▽—) on days 2, 7, 12 and 17 before 2 days of Hsp70 immunization (B.) as described in Materials and methods. Thereafter, the spleen cells were harvested and incubated with DL-cells at the effector/target cell ratio as indicated for 5 days, and MTT assay was performed. The absorbance was read at 540 nm and the result is presented as mean absorbance ± SE_M of at least three experiments in triplicate.

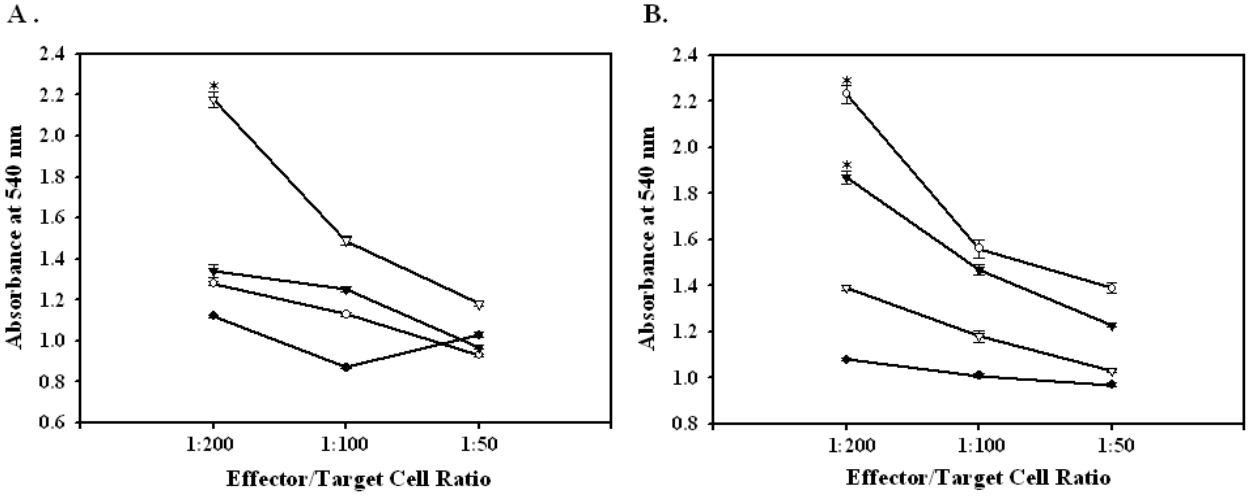

Fig. 6. Effect of autologous Hsp70 immunization and anti-CD4/CD8 treatment on cytotoxic activities of spleen cells against S-180 cells. After S-180 cell transplantation, mice were injected *i.p.* with sterile PBS (—●—), 50 µg autologous Hsp70 (—▽—) or Hsp70 derived from healthy liver (—○—) or Hsp70 derived from syngenic tumor (—▼—) on days 4, 9, 14 and 19 (A.) and treated with sterile PBS (—●—), autologous Hsp70 (—○—), autologous Hsp70 + anti-CD4 antibody (—▼—) and autologous Hsp70 + anti-CD8 antibody (—▽—) on days 2, 7, 12 and 17 before 2 days of Hsp70 immunization (B.) as described in Materials and methods. Thereafter, the spleen cells were harvested and incubated with S-180 cells at the effector/target cell ratio as indicated for 5 days, and MTT assay was performed. The absorbance was read at 540 nm and the result is presented as mean absorbance ± SE_M of at least three experiments in triplicate.

anti-CD8 pretreatment completely block the cytotoxic activities of spleen cells at E/T ratio of 1:200, 1:100, 1:50. It is because of the fact that only $CD8^+$ T lymphocytes contain CTL property not the $CD4^+$ T lymphocytes.

Post-Hsp70 immunization pro-inflammatory serum cytokine level. After the determination of induction of cell-mediated immune response against DL cells and S-180 cells, we then checked for the induction of non-specific immune response induced by pro-inflammatory cytokine in the tumor-bearing host. There are no significant differences for the pro-inflammatory cytokines IFN-γ, TNF-α and IL-2 or the anti-inflammatory cytokines IL-10 and IL-13 in the serum of

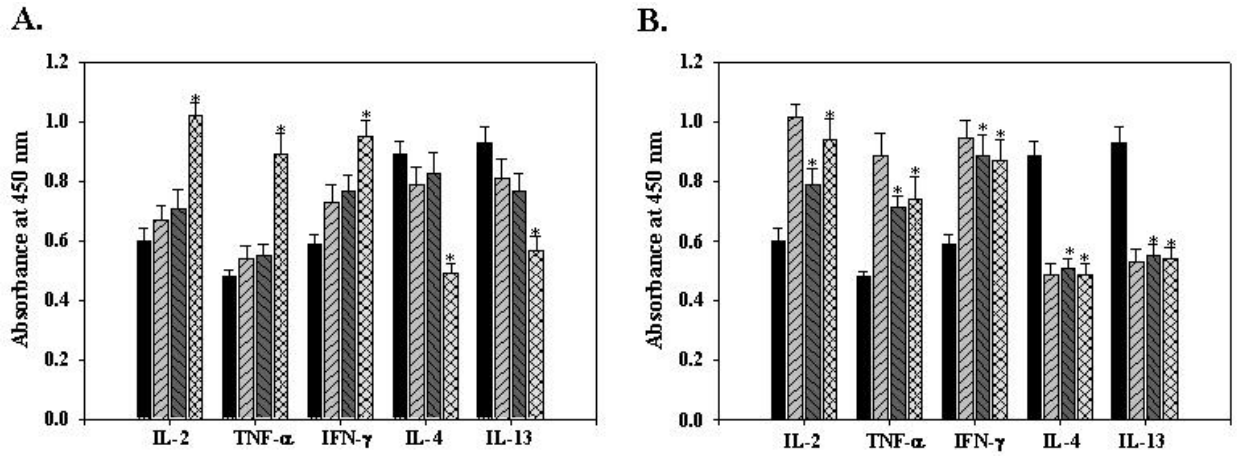

Fig. 7. Post-Hsp70 immunization pro-inflammatory serum cytokine level in DL-bearing mice. After DL-cell transplantation, mice were injected *i.p.* with sterile PBS ( ), 50 µg autologous Hsp70 ( ) or Hsp70 derived from healthy liver ( ) or Hsp70 derived from syngenic tumor ( ) on days 4, 9, 14 and 19 (A.) and treated with sterile PBS ( ), autologous Hsp70 ( ), autologous Hsp70 + anti-CD4 antibody ( ) and autologous Hsp70 + anti-CD8 antibody ( ) on days 2, 7, 12 and 17 before 2 days of Hsp70 immunization (B.) as described in Materials and methods. Thereafter, serum was prepared and cytokine quantification was performed by ELISA. The absorbance was read at 450 nm and the result is presented as mean absorbance ± SE_M of at least three experiments in triplicate.

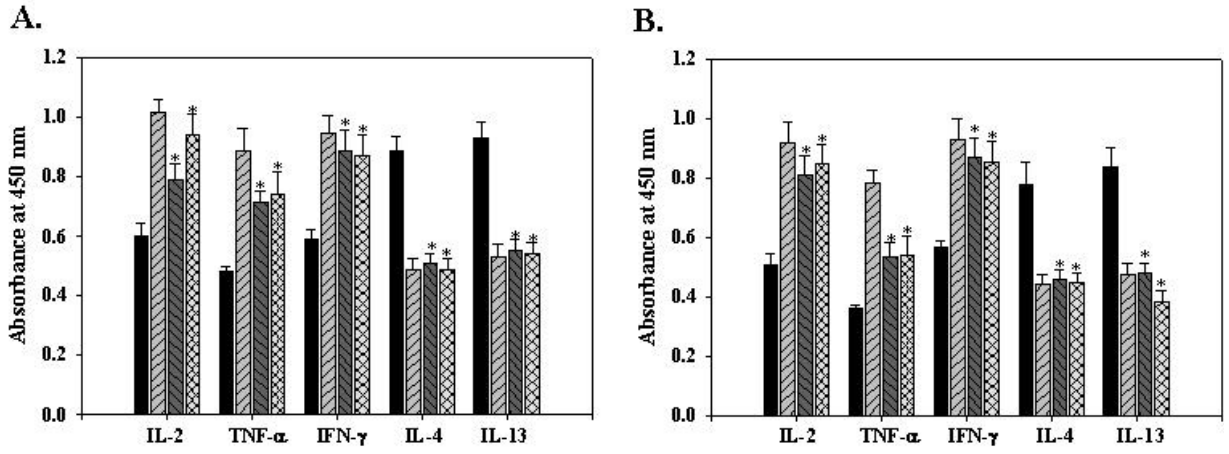

Fig. 8. Post-Hsp70 immunization pro-inflammatory serum cytokine level in mice bearing sarcoma. After S-180 cell transplantation, mice were injected *i.p.* with sterile PBS ( ), 50 µg autologous Hsp70 ( ) or Hsp70 derived from healthy liver ( ) or Hsp70 derived from syngenic tumor ( ) on days 4, 9, 14 and 19 (A.) and treated with sterile PBS ( ), autologous Hsp70 ( ), autologous Hsp70 + anti-CD4 antibody ( ) and autologous Hsp70 + anti-CD8 antibody ( ) on days 2, 7, 12 and 17 before 2 days of Hsp70 immunization (B.) as described in Materials and methods. Thereafter, serum was prepared and cytokine quantification was performed by ELISA. The absorbance was read at 450 nm and the result is presented as mean absorbance ± SE_M of at least three experiments in triplicate.

tumor-bearing host at day 0 of tumor cell transplantation from the normal healthy mice or control group of mice. However, in the tumor-bearing host, the anti-inflammatory cytokines were found to increase, while pro-inflammatory cytokine level did not alter. Strikingly, there was increase in the level of pro-inflammatory cytokines in the serum of the Hsp70 immunized tumor-bearing mice, whereas the level of anti-inflammatory cytokines was found to decrease in the milieu (Fig. 7A & 8A). T lymphocytes depletion reduces the level of pro-inflammatory cytokines whereas in did not affect the level of anti-inflammatory cytokines in both the tumor models (Fig. 7B & 8B). It indicates that Hsp70 immunization, in addition to cell mediated immunity, increases pro-inflammatory cytokines in the milieu of tumor-bearing host.

Discussion

Heat shock proteins such as Hsp70, Hsp90 and gp96 enhance immune responses and are clinically used for immunotherapy of malignant tumors [11, 19, 34–38]. In the present study, we have demonstrated that immunization of tumor cell derived Hsp70 induced an effective survival advantage in mice with minimal residual tumor cells to which Hsp70 is derived. In addition, we revealed the involvement of immune cell types in the rejection of tumor in the Hsp70 immunized tumor-bearing host mice and their post-immunization cytokine repertoire.

Our results demonstrate that autologous Hsp70 induces specific anti-tumor immunity and effectively eradicates tumors in the host mice, thus enhances survival of tumor-bearing host. Our results also indicate that $CD4^+$ and $CD8^+$ T lymphocytes are involved in the induction of anti-tumor immune responses. Previously, it has been reported that antigenic peptide with an Hsp70-binding domain complexed to Hsp70 can enhance the potency of CTL induction and *in vivo* immunity [39]. Here in this case, the antigenic peptides chaperoned by Hsp70 are derived from cellular proteins of DL cells and S-180 cells. This antigenic repertoire may consist of peptides that are rendered antigenic by the mutations [40]. Immunization with tumor-derived Hsp70 preparations permits antigen presenting cells (APCs) to present tumor antigen to their cell surface [41] through the endogenous pathway of antigen presentation [21]. It indicates that HSP70-peptide complexes, even though provided exogenously, are able to channel the tumor antigenic peptides into the class I presentation pathway. The blocking of CTL response after anti-CD8 treatment also confirms that the tumor antigenic peptide is channeled into the class I presentation pathway. Hsp70 from either syngenic tumors or Hsp70 derived from normal liver did not show the prolongation of survival days in the tumor-bearing host. Furthermore, permanent Hsp70 over expression either by exogenous application or by induction of hyperthermia augments MHC class I cell surface expression [42, 43], and thereby further intensifying the lysability of cytotoxic $CD8^+$ T lymphocytes.

Treatment with anti-CD4 did not significantly block the tumor cell lysis. However, they are equally required for the prolongation of survival by Hsp70 immunization, which indicates that some antigenic peptides are also presented on MHC class II and are recognized by $CD4^+$ T lymphocytes. But, $CD4^+$ T lymphocytes do not directly induce CTL immune response, instead after encounter with the presented tumor antigen; they are activated and activated $CD4^+$ T lymphocytes secrete type I cytokines such as IL-2, IFN-γ, and TNF-α, and indirectly induce the cytotoxic $CD8^+$ T lymphocytes as proposed by Sato et al.[30]. In our study, increase in type I cytokines in the serum of Hsp70 immunized mice confirms this assumption. In addition, it has been well established that Hsp70 stimulate the production of cytokines from mononuclear cells, macrophages and dendritic cells [44–46], which might be another method to enrich host's internal milieu with type I cytokines after Hsp70 immunization. Furthermore, we have previously demonstrated that exogenous application of Hsp70 restore tumor-associated macrophages (TAMs) production of Th 1 polarizing cytokines and induce the production of proinflammatory cytokines IFN-γ, IL-12 and IL-15 [27]. However, the pro-inflammatory serum cytokine level in both the tumor system varies. It confers additional advantage to the anti-tumor cytokine $CD8^+$ T cell responses.

It has been reported that mice administered with dendritic cells pulsed with A20-derived peptides and fibroblasts expressing CD40 ligand and secreting IL-2 showed a systemic disorder resembling graft-versus-host reaction [47]. In our study, Hsp70 immunized mice did not show any such systemic disorder such as weight loss, loss of fur etc. Therefore, it could be assumed as safe and might be clinically useful for vaccination against malignant tumors such as in present study, Dalton's lymphoma and sarcoma mice tumor model. However, further studies are required to generalize these observations in this regard to other type of human malignancies.

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