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*Full Length Research Paper*

# Reduced treg and onset of a $T_H$1pattern in combined HSP70 and propranolol treatment of fibrosarcoma-bearing mice

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**Experimental strategies for cancer treatment have been developed to enhance cell-mediated immunity; some have generated promising results. Several heat shock proteins (HSP) derived from tumorgenic cells have been found capable of effectively initiating specific anti tumor immunity. The sympathetic nervous system innervates primary lymphoid organs and also influences T-lymphocyte maturation. This study had two main objectives: first to evaluate the effects of HSP-70-rich tumor lysate as an experimental tumor treatment and secondly, to assess whether by promoting anti-tumor cell-mediated immune responses, propranolol could improve cancer therapy outcomes. In this study, female BALB/c mice and mouse fibrosarcoma cells were used to establish a tumor model. After treatment with HSP70-rich lysate, with or without pronpranolol co-treatment, splenocyte proliferation was evaluated using ELISA BrdU kits. The ability of the treatments to shift the cytokine profile was evaluated by measuring host splenocyte IFN and IL-4 production *ex vivo*. The frequency of T-regulatory ($T_{reg}$) cells in the spleen was analyzed using anti-CD4, anti-CD25, and anti-Foxp3 triple-color immunostaining. Assessments of cytotoxic T-lymphocyte (CTL) activity and *in situ* tumor growth were also performed. The results showed that, compared to the untreated control group, a decrease in tumor size, IL-4 production, and levels of splenic $CD4^+CD25^+Foxp3^+$ $T_{reg}$ cells was observed as a result of the propranolol + HSP70 co-treatment. A significant increase in IFNγ formation was also noted. This study confirmed our hypothesis that parallel administration of HSP70-enriched lysate and propranolol could reduce tumor size *in situ* through increases in IFNγ and decreases in IL-4, in part via an augmentation of the host $T_H$1-type immune response.**

**Key words:** Immune modulation, sympathetic nervous system, heat shock proteins (HSP), tumor-antigen-specific CTL.

## INTRODUCTION

Several experimental strategies in animal models have been developed to evaluate the enhancement of anti-cancer cell-mediated immune responses. In addition, several Phase I and II clinical trials using these vaccine strategies have shown extremely encouraging clinical results (1)(Chen and Wu, 1998).

Heat shock proteins (HSPs) are intracellular proteins that act as antigen chaperones. When a cell is subjected to temperature changes, HSPs bind to intracellular peptides and chape-rone a large number of non-defined antigenic peptides derived from the cells (2,3). Some HSPs derived from cancer cells have been found to effectively initiate specific immunity through attachment and transpor-

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tation of antigenic peptides to antigen-presenting cells (APC) to subsequently activate tumor-antigen-specific cytotoxic T-lymphocytes (CTL) (4-8) . Clinical trials using tumor-derived HSPs have been conducted in patients with a broad range of malignancies, including lymphoma, renal cell carcinoma, melanoma, colorectal cancer, gastric cancer, pancreatic cancer and breast cancer (9,10) .

It is well documented that the sympathetic nervous system, a major component of the autonomous nervous system, innervates primary lymphoid organs (i.e., thymus and bone marrow) (11-13). The expression of β-adrenergic receptors ( β-AR) has been revealed on the surface of both thymo-cytes (14, 15) and thymic non-lymphoid cells (13, 16-18). Accordingly, it has been suggested that noradrenaline (NA), the principal neurotransmitter released from sympathetic nerve terminals, influences T-lymphocyte maturation, not only directly via β-AR on the developing lymphocytes, but also indirectly by acting on the thymic non-lymphoid cells via β-AR (18 ). However, knowledge about the role of β-AR-mediated signaling in the modulation of intra-thymic T-lymphocyte development is still extremely limited. Since it has been shown that thymocytes express a significantly lower number of β-AR on their surface in comparison with circulating peripheral T-lymphocytes (19-20 ), it is assumed that surface expression of β-AR increases during T-lympho-cyte maturation. Furthermore, it has been speculated that stimulation of β-AR may impede lymphocyte development, and that lowering of β-AR expression on developing lymphocytes is a beneficial phenomenon (21).

*In vitro* studies have revealed that NA influences the expression of Thy-1 antigens on fetal mouse thymic stem cells (22). By analyzing TCR β and CD4/CD8 co-receptor expression in adult male Wistar rats subjected to a long-lasting Substance P treatment, Leposavi´c et al. were able to conclude that β-AR blockade influences T-lymphocyte differentiation and consequently, T-lymphocyte-dependent functions(23).

The aim of this study was to assess the effects of propranolol on the efficacy of an HSP-70-rich lysate in immunotherapy against fibrosarcoma. To achieve this goal, mice were injected with fibrosarcoma tumor cells and then treated with/without lysate from heat-shocked WEHI-164 tumor cells.

## MATERIALS AND METHODS

### Mice

Six-week-old female BALB/c mice were purchased from the Pasteur Institute (Tehran, Iran). All mice were housed in pathogen-free facilities maintained at 22°C with a 40% relative humidity and a 12-hr light/dark cycle. All mice had *ad libitum* access to sterilized water and autoclaved standard mouse chow throughout the study. The animal protocol was reviewed and approved by the Animal Care and Research Committee of the Tarbiat Modares University**.**

### Cell culture

BALB/c mouse fibrosarcoma cells (WEHI-164) were obtained from the Pasteur Institute and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) at 37°C incubator containing 5% $CO_2$ and 95% humidity.

### Induction of HSP-70 in WEHI-164 cell line

Cells in logarithmic growth phase were heated by direct immersion of the culture flasks in a waterbath at 42°C for 60 min. After the heat treatment, the cells were incubated at 37°C for 8 and 12 hr and then harvested using trypsin/EDTA (Gibco). The cells were washed three times with phosphate-buffered saline (PBS, pH 7.4; centrifugation at 1200 rpm, 5 min) and finally re-suspended in PBS at $5 \times 10^6$ cells/ml. These HSP70-enriched cells were then used to prepare HSP70-enriched lysates for host treatment as indicated below. Cells without the heating step were used to prepare non-enriched WEHI lysate for use in host treatments as indicated.

### Lysate preparation

Tumor cell lysates (enriched and non-enriched forms) were prepared according to earlier methods (24,25). Briefly, cell suspensions ($5 \times 10^6$ cells total) in PBS were disrupted by five freeze–thaw cycles using liquid nitrogen and a 37°C waterbath. After the final cycle, any large particles were removed by centrifugation (20 min, 3000 rpm, 4°C) and the resultant supernatant was passed through a 0.2-µm filter (Millipore, Billerica, MA). The protein concentration in each sample was determined using the microbradford method. The prepared lyastes were used for treatment of the tumor-bearing mice (with/without propranolol).

### Tumour antigen preparation

A total of $10^7$ WEHI-164 cells were subjected to sonication (60 HZ, 0.5 amplitude). Phenylmethane sulfonyl fluoride (PMSF, 1 mM) was added (as 50 µl aliquot) prior to sonication to inactivate any proteinases present. Thereafter, the protein concentration in each sample was determined using the Bradford method; the remaining was stored at -20ºC for future use in splenocyte

proliferation and cytokine production assays.

**Tumor models and exposure protocols**

WEHI-164 cells were prepared to a final concentration of 5 x $10^6$/ml as noted above. To initiate tumor growth *in situ*, 5 x $10^5$ cells (100 µl) were injected subcutaneously into the right flank of the mice (26). Once the tumors had been established (i.e., on Day 14 post-injection), mice were randomly assigned to one of five experimental groups:
Group 1: Treatment with HSP70-enriched lysate (1.3 mg/mouse);
Group 2: Treatment with HSP70-enriched lysate and 3 mg propranolol/kg;
Group 3: Treatment with 3 mg propranolol/kg only;
Group 4: Treatment with PBS only; or,
Group 5: Treatment with non-enriched lysate (1.3 mg/mouse).
At least 5 mice in each experimental group were injected intraperitoneally with lysate, PBS, propranolol (or in various combinations as indicated) in a total volume of 0.1 ml. For the co-treatments, the injection volumes of the lysate and the propranolol were combined (as 50 µl volumes) to yield the final 100 µl volume. All tumor-bearing mice were treated daily for 20 consecutive days post tumour induction ( a total of 34 days). The dose of lysate was based upon previous experiments performed by our group (27). The propranolol dose was based on doses routinely used in clinic and previous studies (28,29). One day after the final injection (i.e., Day 35 of entire experiment), whole blood was drawn (via retro-orbital puncture) and the mice were then sacrificed by cervical dislocation. At necropsy, the spleen was then collected aseptically for use in the various assays outlined below.

**Splenocyte proliferation**

Splenocytes from the organ recovered at necropsy were isolated in RPMI 1640 via needle perfusion. Erythrocytes present were lysed using lysis buffer (0.8% $NH_4Cl$, 12 mM $KHCO_3$, 0.1 mM $Na_2EDTA$) for 7 min at 25°C. The remaining cells were centrifuged, washed with PBS, and then re-suspended in RPMI 1640 supplemented with 10% heat inactivated FBS, 100 µg streptomycin/ml, 100 U penicillin/ml, 2 mM L-glutamine, and 25 mM HEPES (without phenol red indicator). After cell viability assessment via trypan blue exclusion, the cell concentration was adjusted to 3 x $10^6$ cells/ml and 100 µl aliquots were seeded into wells of 96-well plates (in triplicate). Prepared tumor antigen was added (to a final level of 25 µg/ml, 20 µl) and the plates were then incubated at 37°C for 36 hr. At the end of this period, 10 µl bromodeoxyuridine (BrdU) labeling solution was added to each well to permit quantification of cell proliferation. Uptake of BrdU was detected using an ELISA kit (Roche Diagnostic GmbH, Mannheim, Germany). Absorbance (OD) values at 450 nm were recorded for each well using a MultiScan MS plate reader (Labsystems, Vantaa, Finland) and the stimulation index (SI) was calculated as: SI = 100 x [(T-N)/(P-N)], wherein T = sample OD, N = negative control OD, and P = positive control OD.

**Splenic mononuclear cell (MNC) cytokine production**

To evaluate the effect of HSP70-enriched and non-enriched lysate vaccine on cytokine production by splenic MNC, after treatments, the spleens were removed under sterile conditions and single cell suspensions prepared as above. The MNC present were then isolated using Histopaque (Baharafshan Co., Tehran) and centrifugation at 700 × *g* (15 min, 20°C). The MNC were recovered, washed twice with PBS (10 min, 360 × *g*, 4°C), and re-suspended in RPMI 1640\10% FBS. After assessment of viability (trypan blue exclusion; cell viability was > 90%), 100 µl aliquots each containing 4 × $10^5$ cells were placed in 96-well microtiter plates. To stimulate the cells, tumor antigen was added (10 µl aliquots) at 5 µg/ml (final concentration). The cells were then incubated for 72 hr at 37°C. At the end of this period, the supernatants in each well were collected and stored at -80°C until analyzed for cytokine contents.
ELISA kits (R&D Systems, Minneapolis, MN) were used to measure interferon (IFN)-γ and interleukin (IL)-4. Following the manufacturer protocols, absorbance values (at 450 nm) were ultimately measured using the MultiScan MS plate reader. IFNγ and IL-4 levels in each sample were determined by extrapolation from a standard curve generated in parallel using kit-provided standards. The levels of detection of IFNγ and IL-4 kits were 2 pg/ml each.

**Cytotoxic T-Lymphocyte (CTL) activity following vaccine therapy**

Splenocyte suspensions were prepared as above in RPMI 1640 (containing 2% bovine serum albumin [Sigma]) as effector cells; mouse WEHI-164 (H-$2^d$) cells were prepared for use as target cells. Briefly, 2 x $10^4$ effector cells (in 100 µl volume per well) were incubated in 96-well plates and pulsed overnight with a 20 µl aliquot of tumor antigen (containing 20 µg tumor antigen/ml). Thereafter, for the CTL assay, fixed volumes of effector cells were transferred to wells containing 100 µl of target cells to establish effector:target (E:T) ratios of 100:1, 50:1, and 25:1. The plates were then gently centrifuged (250 x g, 10 min) and placed in a 37°C incubator for 4 hr. The plates were then centrifuged and 100 µl supernatant from each well was transferred to a 96-well flat-bottom

plate; the extent of cytotoxicity that had occurred was then determined by assaying LDH release with an LDH kit (Takara Company, Tehran) according to manufacturer protocols and absorbance measurements at 492 and 620 nm in the MultiScan plate reader. Specific lysis (%) was calculated as: 100 x (LDH release in sample well – spontaneous LDH release by effector cells – spontaneous LDH release by target cells)/(maximum LDH release by target cells – spontaneous LDH release by target cells). All determinations were performed in triplicate. Maximum lysis was determined from supernatants of cells lysed with 1% Triton X-100; spontaneous release was determined from target cells incubated with RPMI 1640\2% BSA only.

### Flow cytometric analysis of T-regulatory ($T_{reg}$) cells in spleen

Freshly-prepared spleen cells were analyzed using direct immunofluorescence staining. Antibodies used for the staining were: fluorescein isothiocyanate (FITC)-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CD25, and PE-Cy5-conjugated anti-Foxp3 (eBioscience, San Diego, CA) according to manufacturer protocols. Briefly, staining was performed in wash-ing buffer (PBS supplemented with 1% heat-inactivated FBS, 0.1% sodium azide, and 2 mM EDTA). Each sample (1 x $10^5$ cells in 100 µl) was treated with 10 µl of each antibody (separately, followed by a washing step) for 45 min at 4°C in the dark. The cells were then repeatedly washed in buffer and the final cell pellet fixed in 2% paraformaldehyde. Flow cyto-metric analysis was then done in an EPICS flow cytometer (Beckman Coulter, Brea, CA). Based on forward and side-scatter patterns, using provided software, lymphoid areas were identified and the various single-, double-, and triple-stained cells were analyzed. A minimum of 100,000 events per sample was acquired each time.

### Tumor volume measurement

Throughout the experiment, tumor volume was monitored using Vernier calipers (Mitutoyo, Tokyo, Japan). Tumor volume (V) was calculated as V($mm^3$) = (π/6) x LWD, where L = length, W = width, and D = depth (30).

### HSP-70 expression levels in blood

The serum samples from each mouse was assayed for HSP70 using a Quantikine ELISA Kit (R&D Systems), according to manufacturer instructions. HSP70 levels in each sample were calculated by extrapolation from a standard curve generated in parallel using kit-provided standards. Kit sensitivity was 90 pg HSP70/ml.

### Statistical analysis

The results were depicted as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA). A p-value less than 0.05 was considered statistically significant.

## RESULTS

### Induced expression of HSP70 with heat treatment

Figure 1 illustrates the levels of HSP-70 in the supernatants of lysate from heat-treated and non-treated WEHI cells. The results indicated a significant increase in the level of HSP-70 in cells after heat treatment compared with non-heat shocked cells (i.e., 1.09 vs. 0.31 mg/ml). The increased expression was statistically significant (p value: 0.02).

### Lymphocyte proliferation index following therapy

To assess lymphocytic activation, after the final injection treatment with HSP70-enriched or non-enriched lysate, spleen cells were collected, cultured, and ‘re-stimulated’ *ex vivo* with tumor antigen. The results shown in Figure 2 indicate that splenocyte proliferative responses of propranolol-treated animals had increased relative to mice in the other treatment groups, though the changes were not significant. In fact, the level of proliferation by cells from mice receiving lysate only (Group 2) was not significantly different from those of mice that received PBS or WEHI lysate only (Groups 4 and 5).

### Cytokine pattern shift following therapy

To ascertain if there was any $T_H1/T_H2$ cytokine shifting in the tumor-bearing mice treated with HSP70-enriched and non-enriched lysate, splenic MNC from mice in each treatment group were isolated and ‘re-stimulated’ *ex vivo* with tumor antigens. ELISA was used to quantify the levels of IFNγ and IL-4 in the culture supernatants. As depicted in Figure 3A, an increase in IFNγ levels was observed among mice that received the HSP70-lysate + propranolol in compari-son to tumor-bearing hosts that received only PBS. IFNγ production/release by MNC did not differ between cells from control group mice and hosts that received either HSP70-non-enriched lysate or propranolol alone.

In contrast, MNC from mice treated with propranolol alone seemed to display a strong decrease in IL-4 formation relative to other group. However, there were no statistical significant changes in IL-4 production among the experimental groups (Figure 3B).

### Cytotoxic T-Lymphocyte (CTL) activity following therapy

The cytotoxic activity of splenic T-lymphocytes was asse-

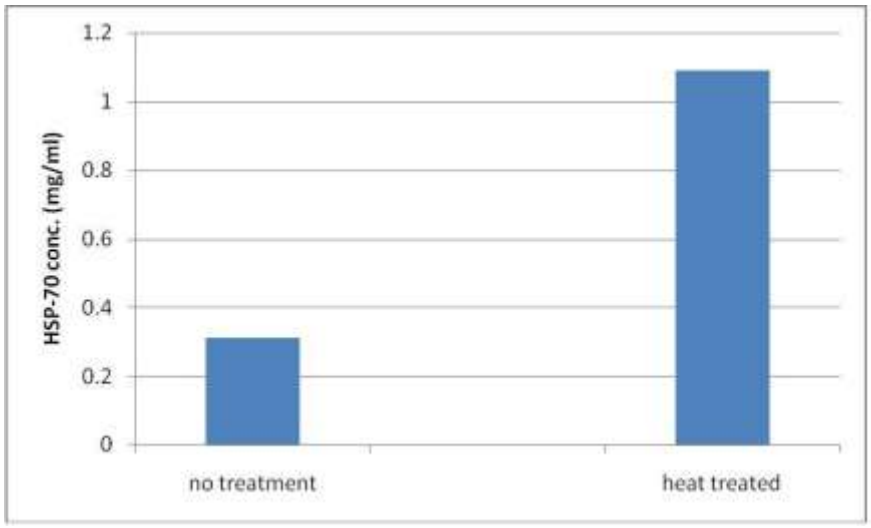


**Figure 1. Levels of HSP-70 expression in the lysate of heat treated WEHI-164 cells.** Induction of HSP-70 following heat treatment of cultured cells at 42°C for 60 min was evaluated using ELISA. As illustrated, the heat treatment was able to induce the expression of HSP70 (i.e., 0.31 vs. 1.09 mg/ml) in the cells.

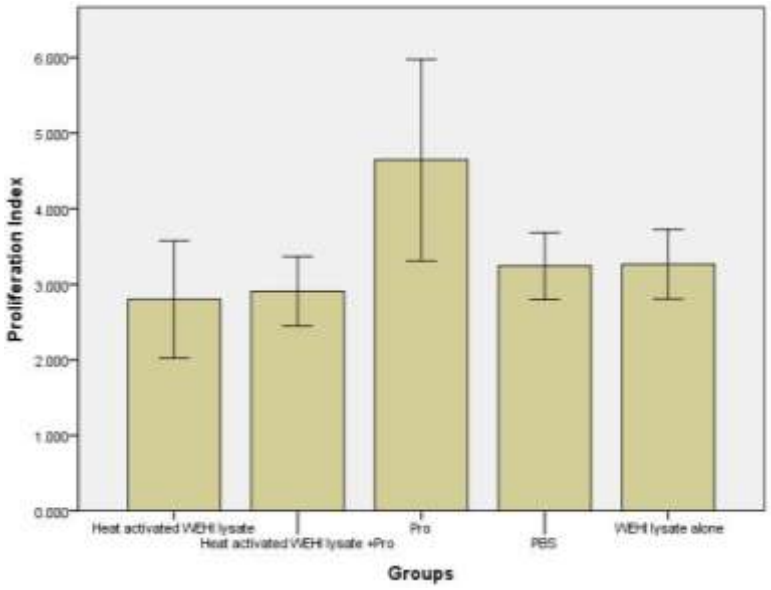


**Figure 2. Splenic lymphocyte proliferation index in treated tumour bearing mice with propranolol and HSP-70 rich lysate.** Splenocytes were recovered from tumor-bearing mice that were treated with HSP70-enriched lysate (1.3 mg/mouse), HSP70-enriched lysate and 3 mg propranolol/kg, 3 mg propranolol/kg only, PBS only, or non-enriched lysate (1.3 mg /mouse). All values are derived from BrdU ELISA measurements. Values shown are mean (± SD) from 5 mice/group. Splenocyte proliferative responses in propranolol-treated animals showed a relative increase to that by cells from mice receiving non-HSP70-bearing lysate (alone or with propranolol), though the changes were not significant. Abbreviations: pro:propranolol treatment, PBS: phosphate buffer saline treatment.

ssed using a target cell killing assay and measures of LDH release. The results in Figure 4 illustrate that the splenocytes from mice in the HSP70-enriched lysate + propranolol group tended to have the greatest killing activity of all, albeit that this effect was least evident at the lowest E:T ratio (i.e., 25:1). Focusing on the 100:1 ratio assay systems, it was clear that this value was significantly greater than that by cells from any other group. Cells from hosts that received the HSP70-lysate alone had the next highest level of activity, but it was not significantly greater than that by cells from hosts that received unmodified WEHI lysate. The levels of CTL acti-

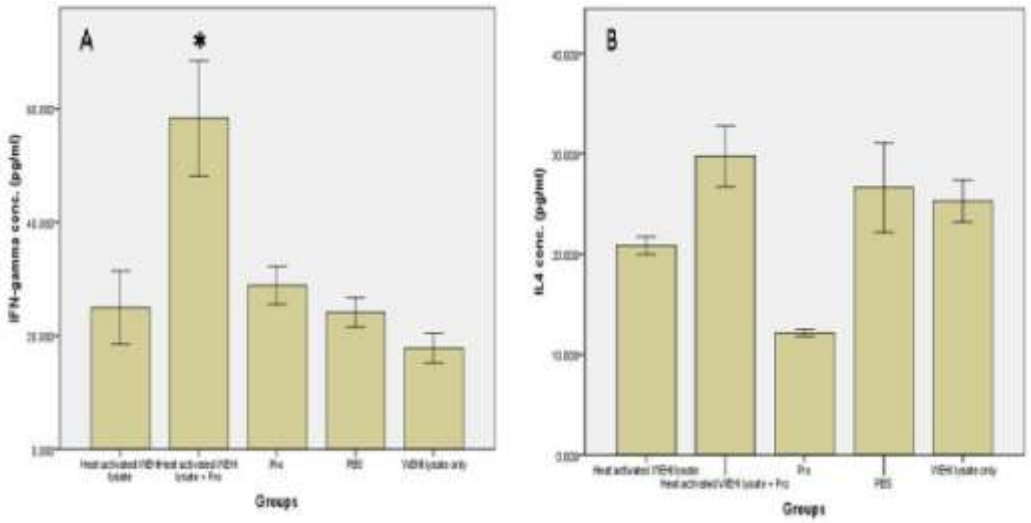


**Figure 3. *Ex vivo* IFN□ and IL4 production by splenic MNC cultures extracted from treated mice**. **(A)** IFN□ and **(B)** IL-4 levels in cultures of splenic MNC recovered from tumor-bearing mice that were treated with HSP70-enriched lysate (and with or without co-treatment with propranolol), non-enriched lysate, propranolol only, or PBS. Values shown are mean (± SD) from 5 mice/group. All tests were evaluated in triplicate. Co-treatment with propranolol was able to induce significant ($p = 0.015$) increases in IFNγ production relative to that by MNC from mice that received HSP70-enriched lysate only, as well as from mice in all the other groups. There were no significant differences among the MNC from the other treatment groups. Decreases in IL-4 production were evident in MNC from mice that received propranolol only, although the drop was not significant. There were no significant differences among other four groups. Abbreviations: pro:propranolol treatment, PBS: phosphate buffer saline treatment.

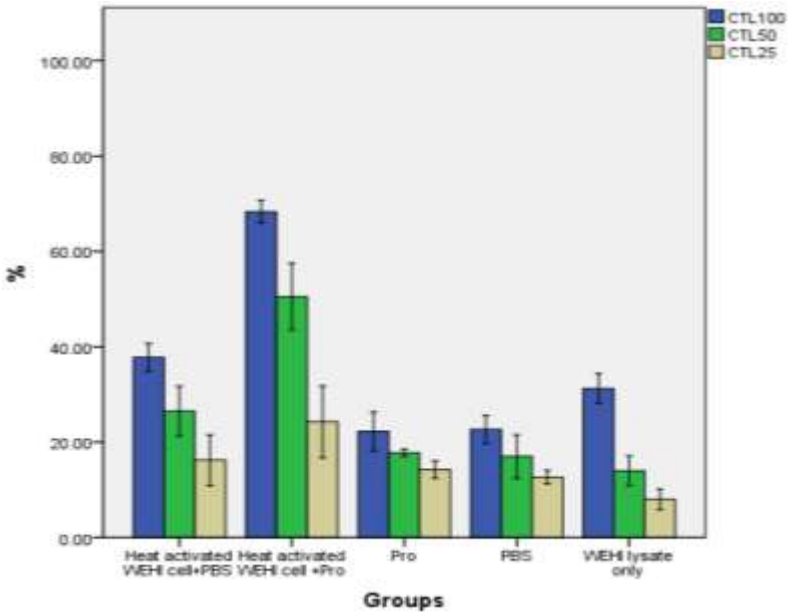


**Figure 4. Cytotoxic activity of splenic T-lymphocytes (CTL activity) from mice treated with propranolol and HSP-70 rich lysate.** Spleens were recovered from tumor-bearing mice in each treatment group and assessed for CTL activity. All values are derived from measures of target cell LDH release. Values shown are mean (± SD) from 5 mice/group. All samples were evaluated in triplicate. Differences were significant in 1:50 and 1:100 ratios with the group receiving co-treatment showing the greatest cytotoxicity compared to other treatments at same E:T ratio ($p < 0.01$). Abbreviations: pro:propranolol treatment, PBS: phosphate buffer saline treatment.

vity by cells obtained from the propranolol only, and PBS controls did not significantly differ from one another.

### Frequency of $CD4^+CD25^+Foxp3^+$ T ($T_{reg}$) cell following vaccine therapy

To assess the frequency of splenic $CD4^+CD25^+FoxP3^+$ $T_{reg}$ lymphocytes in the tumor-bearing hosts, splenocytes were isolated and flow cytometry was performed. Based on dot-plots, CD25 and Foxp3 co-positive cells gated in lymphocytes and $CD4^+$ cells, it was determined that all of the experimental groups had fewer numbers of splenic $T_{reg}$ populations relative to levels in mice that had received PBS only or HSP70-enriched lysate alone (Figure 5). $T_{reg}$ cell

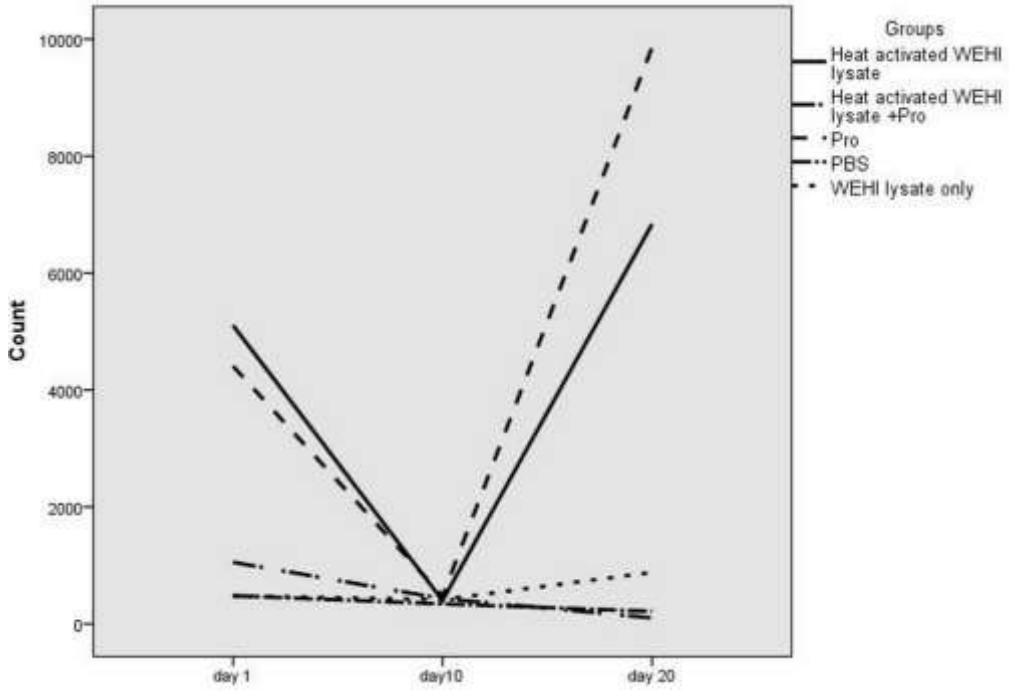


**Figure 5. Splenic $T_{reg}$ cell levels after treatment with propranolol and HSP-70 in tumour bearing mice.** Freshly-prepared spleen cells were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD25, and PeCy5-conjugated anti-Foxp3. Based on dot-plots, CD25 and Foxp3 co-positive cells gated in lymphocytes and $CD4^+$ cells, it was determined that all the experimental groups had fewer numbers of splenic $T_{reg}$ cells relative to levels in mice that had received the PBS only treatment; with mice that received the heat-activated cell lysate, the difference from the control was significant ($p = 0.02$). Abbreviations: pro:propranolol treatment, PBS: phosphate buffer saline treatment.

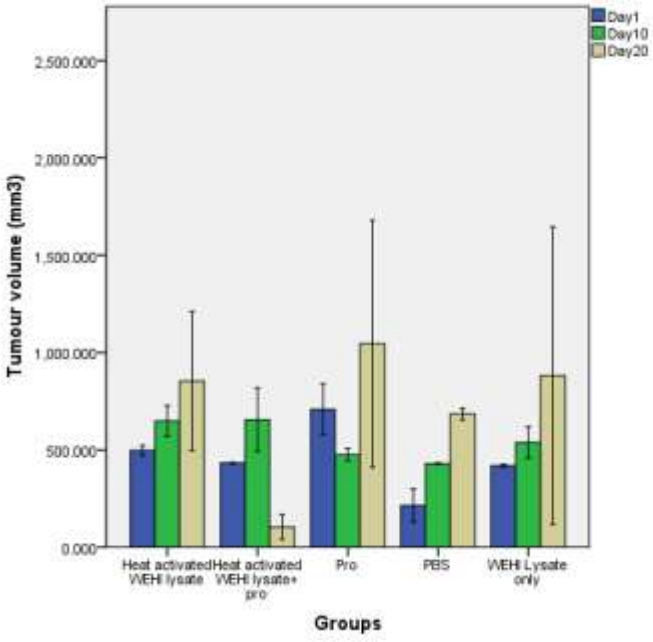


**Figure 6. Tumor size in mice during course of treatment.** Tumor volume (in $mm^3$) was calculated and monitored throughout the experiment. The results indicate that tumors in mice receiving HSP70-rich lysate ± propranolol grew more slowly than those in the control groups. Co-treatment (HSP70-enriched lysate + propranolol) caused a more effective growth control over the 20-d monitoring period compared to either single treatment (lysate or propranolol alone) or no treatment (PBS or lysate only). Propranolol itself was able to partially control growth until Day 10; however thereafter, tumor growth progressed. Abbreviations: pro:propranolol treatment, PBS: phosphate buffer saline treatment.

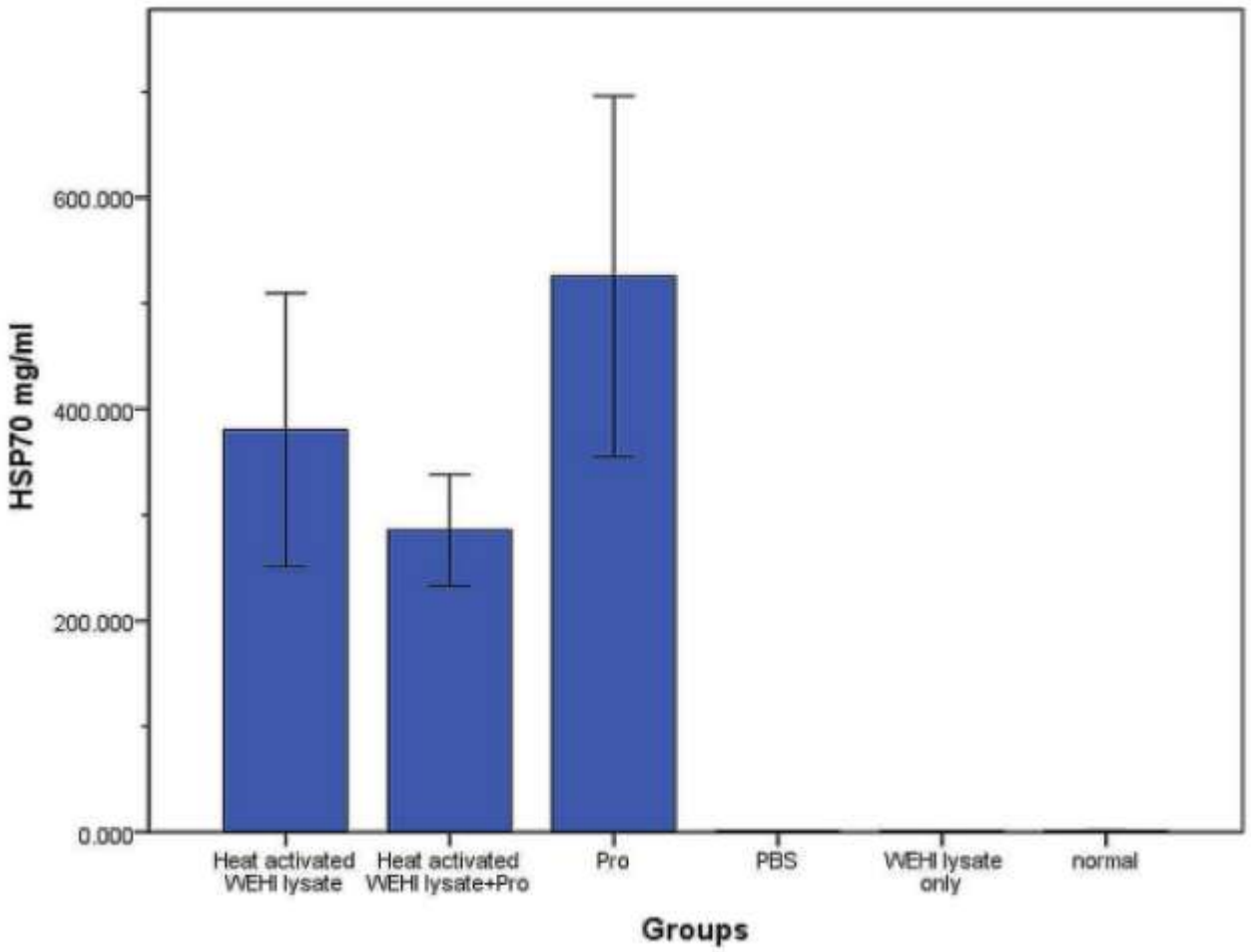


**Figure 7.** Level of HSP70 in sera of mice treated with propranolol and HSP-70 rich lysate. Blood was drawn at sacrifice and serum was obtained and assayed for HSP70 by ELISA. Values shown are mean (± SD) from 5 mice/group. All samples were evaluated in triplicate. *Value significantly different from controls receiving PBS and also from mice treated with non-enriched lysate ($p < 0.01$). No significant differences were observed among treatment Groups 1, 2 and 3. Abbreviations: pro:propranolol treatment, PBS: phosphate buffer saline treatment.

levels were significantly lower among splenocytes from mice that had received the HSP70-enriched lysate + propranolol regimen or propranolol alone. However, ultimately, the levels of $T_{reg}$ cells did not significantly differ among these three groups.

**Tumor growth *in situ***

Changes in tumor growth in each experimental group were assessed by weekly measures of tumor mass with a digital caliper. The results (Figure 6) indicate that the tumors in the mice receiving HSP70-rich lysate with or without the co-treatment with propranolol grew more slowly than those in the PBS control mice (compare changes from Day 1 to Day 10 to Day 20). A similar trend was apparent among hosts that received propranolol alone; however, the somewhat surprising data on Day 10 makes interpretation of this trend somewhat confusing. Ultimately, by Day 20 of the treatment regimen, mice that had received the HSP70-enriched lysate of heat-shocked tumor cells (in conjunction with propranolol) displayed significant ($p = 0.06$) reductions in tumor size over the final 10 days of measurements.

**Systemic levels of HSP70 following vaccine therapy**

To assess the microenvironment of the spleen in the mice treated with HSP70-enriched and non-enriched lysate with or without propranolol, sera was analyzed for HSP70 using the same ELISA used to assess splenocyte levels (see above). The results illustrated in Figure 7, indicate that there was, as expected, a significant increase in the level of HSP70 in the sera of mice treated with HSP70-enriched lysate (with or without propranolol). Mice that received only PBS or non-shocked WEHI cells

did not show any increase in circulating levels of HSP70. Oddly, treatment with propranolol alone significantly increased the level of HSP70 as well and also caused a repression in mice that were co-treated with the HSP70-enriched lysate.

## DISCUSSION

The peripheral nervous system is one of the factors that contribute to wound healing, a dynamic process that includes inflammation, granulation tissue formation, contraction, and re-epithelialization (31). Increased levels of neuropeptides and their respective receptors are observed in tissues during inflammation (32). Neuropeptides present a wide range of effects in inflammatory phase of wound healing. Substance P, a neuro-peptide present in the small-diameter primary afferent nerves, stimulates mononuclear and polymorphonuclear leukocyte activation (33). After injury, the cutaneous nervous terminals are stimulated and produce anti-dromic impulses. These impulses cause the secretion of neuropeptides along peripheral nerves, which will induce neurogenic inflammation characterized by vasodilatation, glandular secretion, and resident inflammatory cell activation (34,45).

Tumor-bearing mice spontaneously lose weight 8-9 weeks after implantation of a human hypernephroma, in spite of a normal food intake. Resting oxygen consumption was up to 40% higher in these animals than in sham-operated controls, but was significantly reduced by □-adre-nergic blockade with propranolol in the former group. The data suggest that the rapid weight loss of tumor-bearing animals may be due to a high metabolic rate that results from sympathetic stimulation of brown-adipose-tissue metabolism (36-38)Anorexia and cachexia accompany advancing cancer to a greater extent than any other symptom. Cachexia alone causes 22% of cancer deaths. Several of the anti-cachectic agents have demonstrated *in vivo* or *in vitro* anti-tumor activity (39). It has been shown that stress hormones significantly increase the invasive potential of cancer cells in vitro (40). Thus it seems that one of the many neglected pathways of cancer invasion can be used for enhancing anti cancer therapies. In this regard, Pasquier et al., in an *in vivo* model of breast cancer has shown that the combination of propranolol and chemotherapy was able to reduce tumour burden. They have shown that these effects are attributed to the anti proliferative and anti angiogenic effects of propranolol (41). Study has shown that co-treatment with propranolol is associated with increased relapse-free survival of breast cancer patients (42,43). It has been shown that beta-adrenergic blockers also exert anticancer effects through non-genomic factors, including matrix metalloproteinase, mitogen-activated protein kinase pathways, prostaglandins, cyclooxygenase-2, oxidative stress, and nitric oxide synthase (44). Also propranolol is able to induce apoptosis in cancer cell lines, decrease EGF and VEGF production via β2-adrenoceptors (45-47).

The profile of cytokine secretion divides T-helper cells into two subpopulations with different roles: the $T_H1$ subset that secretes interleukin (IL)-2 and interferon (IFN)- γ (32,33 Cherwinski et al., 1987; Jeschke et al., 2008) and the $T_H2$ subset that produces IL-4 and IL-5 (33 Cherwinski et al., 1987). IL-4 and IFNγ have modulatory effects on macrophages, which are in some cases coincidental and in others opposing. It has also been found that IL-4 inhibits the production of IFNγ from mononuclear cells. On the other hand, $T_{reg}$ lymphocytes are specialized in the control of responsiveness to self, and are comprised of subsets with distinct ontogeny and functions.

Naturally-occurring $CD4^+CD25^{high}$ $T_{reg}$ lymphocytes are produced in the thymus (48) and express FoxP3, a transcriptional factor critical for their development and function (49,50). Their depletion results in the development of autoimmune diseases in murine models (51,52). $T_{reg}$ lymphocytes are also generated in the periphery from non-regulatory T-lymphocytes (53-55). These include regulatory type 1 (Tr1) (55) and $T_H3$ cells (49), both of which preferentially secrete regulatory cytokines (i.e., IL-10, and/or TGF) and do not express FoxP3 (55,56) . These $T_{reg}$ lymphocytes suppress immune responses in a soluble factor-dependent manner, and do not require cell-to-cell contact. Another $CD4^+$ $T_{reg}$ lymphocyte subset that expresses CD25 and FoxP3 is induced in the periphery *de novo* from FoxP3 non-regulatory T-lymphocytes in the mouse (57-59). $T_{reg}$ lymphocytes inhibit both the development and effector functions of tumor-specific T-lymphocytes. $CD4^+CD25^{high}$ $T_{reg}$ lymphocytes accumulate at the tumor site (60), where they appear to directly suppress cytotoxic T-lymphocyte responses against the tumors (61). Indeed, their depletion permits immune-mediated tumor rejection in murine models of cancer. $CD4^+CD25^{high}$ $T_{reg}$ lymphocytes are also increased in metastatic lymph nodes and peripheral blood of patients with various types of cancers such as metastatic melanoma (62).

In our laboratory, treatment with a combination of HSP70-enriched lysate and proprano-lol effectively decreased the tumor growth. It was initially proposed that propranolol would enhance anti-tumor immune responses via modulation of cytokine production from the lysate-primed immune cells; therefore, the effect of the HSP70-enriched lysate and propranolol on IFNγ and IL-4 production was investigated. It was noted here that splenocytes of HSP70-treated mice, compared to cells from the control groups, had a significant increase in IFNγ production and a decrease in that of IL-4. These outcomes suggested that the propranolol + HSP70-lysate co-treatment is capable of immunomodulation, in part, by inducing a shift in cytokine patterns towards one more aligned with a $T_H1$ (re: IFNγ) pattern in these mice. The precise therapeutic action of propranolol in treatment of cancer

needs further study. The synergistic action of HSP-70 and propranolol and instances of individual effects as reported here, are cases to be studied in more detail to elucidate the mechanism behind their action.

In summary, this study has confirmed that administration of an HSP70-enriched vaccine in conjunction with propanolol could lead to enhanced reduction in tumor size. These outcomes were associated with increased formation/release of IFNγ, and decreased of IL-4, by host's splenocytes levels and an augmentation of the $T_H1$ immune response (in an antigen-specific manner). Moreover, this co-treatment led to increased proliferation of host lymphocytes and enhanced T-lymphocyte function. Further, assessments of splenic $T_{reg}$ cell ($CD4^+CD25^+Foxp3^+$) levels here indicated that use of the vaccine led to a significant decrease in these cells. In the context of tumor immunology, $CD4^+CD25^+Foxp3^+$ T-lymphocytes have a suppressor role during anti-tumor immune responses. Removing these regulatory lymphocytes or blocking immunoregulatory pathways they are responsible for might improve the efficacy of tumor vaccines or the overall immunotherapy against cancer. Indeed, depletion of $T_{reg}$ cells was shown to enhance tumor immunosurveillance, and induced rejection of multiple immunogenic tumors, in several strains of mice (63). While we conclude that a possible mechanism for the anti-tumor activity associated with use of an HSP70-enriched vaccine may be a modulation of the host immune response, further study is warranted before more firm mechanisms can be established.

**Declaration of interest**

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

**REFERENCES**


Chen CH, Wu TC (1998). Experimental vaccine strategies for cancer immunotherapy. J. Biomed. Sci. 5:231-252.

Gething MJ, Sambrook J (1992). Protein folding in the cell. Nature 355:33-45.

Srivastava PK, Udono H (1994). Heat shock protein-peptide complexes in cancer immunotherapy. Curr. Opin. Immunol. 6:728-732.

Tamura Y, Peng P, Liu K, Daou M, Srivastava PK (1997). Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* 278:117-120.

Arnold-Schild D, Hanau D, Spehner D, Schmid C, Rammensee HG, de la Salle H, Schilde H (1999). Cutting edge: Receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. J. Immunol. 162:3757-3760.

Binder RJ, Anderson KM, Basu S, Srivastava PK (2000). Cutting edge: Heat shock protein gp96 induces maturation and migration of $CD11c^+$ cells *in* vivo. J. Immunol. 165:6029-6035.

Basu S, Binder RJ, Ramalingam T, Srivastava PK (2001). CD91 is a common receptor for heat shock proteins gp96, HSP90, HSP70, and calreticulin. Immunity 14:303-313.

Wang XY, Manjili MH, Park J, Chen X, Repasky E, Subjeck JR (2004). Development of cancer vaccines using autologous and recombinant high molecular weight stress proteins. Methods 32:13-20.

Belli F, Testori A, Rivoltini L, Maio M, Andreola G, Sertoli MR, Gallino G, Piris A, Cattelan A, Lazzari I, Carrabba M, Scita G, Santantonio C, Pilla L, Tragni G, Lombardo C, Arienti F, Marchianò A, Queirolo P, Bertolini F, Cova A, Lamaj E, Ascani L, Camerini R, Corsi M, Cascinelli N, Lewis JJ, Srivastava P, Parmiani G (2002). Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96-peptide complexes: Clinical and immunologic findings. J. Clin. Oncol. 20:4169-4180.

Srivastava P (2002). Interaction of heat shock proteins with peptides and antigen-presenting cells: Chaperoning of the innate and adaptive immune responses. Annu. Rev. Immunol. 20:395-425.

Williams JM, Felten DL (1981). Sympathetic innervation of murine thymus and spleen: A comparative histofluorescence and biochemical study. Anat. Res. 199:531-542.

Madden KS, Felten DL (1995). Experimental basis for neural-immune interactions. *Physiol. Rev.* 75:77-106.

Friedman EM, rwin MR (1997). Modulation of immune cell function by autonomic nervous system. Pharmacol. Ther. 74:27-38.

Singh U (1979). Effects of catecholamines in lymphopoesis in fetal mouse thymic explants. J. Anat. 129:279-285.

Marchetti B, Morale MC, Paradis P, Bouvier M (1994). Characterization, expression, and hormonal control of a thymic β2-adrenergic receptor. Am. J. Physiol. 267:E18-31.

B¨orne HR, Lichtenstein TM, Mean KL (1989). Pharmacological control of allergic histamine release *in vitro*: Evidence for an inhibitory role of 3',5'-adenine monophosphate in human leukocytes. J. Immunol. 142:695-705.

Kurz B, Feindt J, von Gaudecker B, Kranz A, Loppnow H, Mentlein R (1997). β-Adrenoceptor-mediated effects in rat cultured thymic epithelial cells. Br. J. Pharmacol. 120:1401-1408.

Sanders VM, Kasprowicz JD, Swanson-Mungerson MA, Podojil JR, Kohm PA (2003). Adaptive immunity in mice lacking the □2-adrenergic receptors. Brain Behav. Immun. 17:55-67.

Pochet R, Delespresse G (1983). Adrenoceptors display different efficiency on lympho-cyte subpopulations.

*Biochem. Pharmacol.* 32:1651-1655.
van de Griend RJ, Astraldi A, Wijermans P, van Doorn R, Ross D (1983). Low adrenergic receptor concentration on human thymocytes. Clin. Exp. Immunol. 51:55-63.
Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES (2000). The sympathetic nerve - an integrative interface between two super-systems: The brain and immune system. Pharmacol. Rev. 52:595-638.
Singh U, Owen JJ (1976). Studies on the maturation of thymus stem cell. The effects of catecholamines, histamine and peptide hormones on expression of T-cell alloantigens. Eur. J. Immunol. 6:59-62.
Leposavi´ CG, Pleca˘s-Solarovi´c B, Kosec D (2000). Differential effects of chronic propranolol treatment on the phenotypic profile of thymocytes from immature and adult rats. Immunopharmacology 46:79-87.
Schnurr M, Galambos P, Scholz C, Then F, Dauer M, Endres S, Eigler A (2001). Tumor cell lysate-pulsed human dendritic cells induce a T-cell response against pancreatic carcinoma cells: An *in vitro* model for assessment of tumor vaccines. *Cancer Res.* 61:6445-6450.
Kim HS, Choo YS, Koo T, Bang S, Oh TY, Wen J, Song SY (2006). Enhance-ment of anti-tumor immunity of dendritic cells pulsed with heat-treated tumor lysate in murine pancreatic cancer. *Immunol. Lett*. 103:142-148.
Panthel K, Meinel KM, Sevil Domenech VE, Geginat G, Linkemann K, Busch DH, Rüssmann H (2006). Prophylactic anti-tumor immunity against a murine fibrosarcoma triggered by the *Salmonella* type III secretion system. Microbes Infect. 8:2539-2546.
Hashemi SM, Hassan ZM, Soudi S, Ghazanfari T, Kheirandish M, Shahabi S (2007). Evaluation of anti-tumor effects of tumor cell lysate enriched by HSP-70 against fibro-sarcoma tumor in BALB/c mice. Int. Immunopharmacol. 7:920-927.
Alcalai R, H Wakimoto (2011). Prevention of Ventricular Arrhythmia and Calcium Dysregulation in a Catecholaminergic Polymorphic Ventricular Tachycardia Mouse Model Carrying Calsequestrin-2 Mutation. J card elecphysi 22(3): 316-324.
Dobarro M, L Orejana (2012). Propranolol reduces cognitive deficits, amyloid β levels, tau phosphorylation and insulin resistance in response to chronic corticosterone administration. Int J Neuropsychopharmacology 1: 1-10.
Zeng Y, Feng H, Graner MW, Katsanis E (2003). Tumor-derived, chaperone-rich cell lysate activates dendritic cells and elicits potent antitumor immunity. *Blood* 101:4485-4491.
Singer AJ, Clark RA (1999). Cutaneous wound healing. New Engl. J. Med. 341:738-746.
Jeschke MG, Finnerty CC, Kulp GA, Przkora R, Mlcak RP, Herndon DN (2008). Combination of recombinant human growth hormone and propranolol decreases hyper-metabolism and inflammation in severely burned children. Pediatr. Crit. Care Med. 9:209-216.
Cherwinski HM, Schumacher JH, Brown KD, Mosmann TR (1987). Two types of murine helper T-cell clone. III. Further differences in lymphokine synthesis between $T_H1$ and $T_H2$ clones revealed by RNA hybridization, functionally mono-specific bioassays, and monoclonal antibodies. J. Exp. Med. 166:129-142.
Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL (1986). Two types of murine helper T-cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136:2348-2362.
Raut SB, Nerlekar SR, Pawar S, Patil AN (2012). An evaluation of the effects of nonselective and cardioselective β-blockers on wound healing in Sprague Dawley rats. Indian J. Pharmacol; 44(5): 629-633
Brooks SL, Neville AM, Rothwell NJ, Stock MJ, Wilson S (1981). Sympathetic activation of brown-adipose-tissue thermogenesis in cachexia. Biosci. Rep. 1:509-517.
Murphy KT, Struk A, Malcontenti-wilson C, Christophi C, Lynch G (2013). Physiological characterization of a mouse model of cachexia in colorectal liver metastases.
Blum D, Omlins A, Baracos VE, Solheim TS, Tan BHL, Stone P, Kaasa S, FearonK Strasser F (2010). Cancer cachexia: A systematic literature review of items and domains associated with involuntary weight loss in cancer.
Davis MP (2002). New drugs for the anorexia-cachexia syndrome. *Curr. Oncol. Rep.* 4:264-274.
Sood AK, R Bhatty (2006). Stress hormone–mediated invasion of ovarian cancer cells. *Clin Can Res* 12(2): 369-375.
Pasquier E, J Ciccolini (2011). Propranolol potentiates the anti-angiogenic effects and anti-tumor efficacy of chemotherapy agents: implication in breast cancer treatment. *Oncotarget* 2(10): 797.
Powe DG, MJ Voss (2010). Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival. *Oncotarget* 1(7): 628.
Bertrand J, C McCuaig (2011). Propranolol versus prednisone in the treatment of infantile hemangiomas: a retrospective comparative study. *Ped derm.* 28(6): 649-654.
Nguyễn LTH (2012). The roles of beta-adrenergic receptors in tumorigenesis and the possible use of beta-adrenergic blockers for cancer treatment: possible genetic and cell-signaling mechanisms. Cancer man res 4: 431
Zhang D, Q Ma (2009). Inhibition of Pancreatic Cancer Cell Proliferation by Propranolol Occurs Through Apoptosis Induction: The Study of [beta]-Adrenoceptor Antagonist's Anticancer Effect in Pancreatic Cancer Cell. *Pancreas* 38(1): 94-100.
Lamy S, MP Lachambre (2010). Propranolol suppresses angiogenesis< i> in vitro</i>: Inhibition of proliferation,

migration, and differentiation of endothelial cells. *Vasc pharm* 53(5): 200-208.

Schuller HM (2009). Is cancer triggered by altered signalling of nicotinic acetylcholine receptors?. *Nat Rev Can* 9(3): 195-205.

Mosmann TR, Coffman RL (1989). $T_H1$ and $T_H2$ cells: Different patterns of lymphokine secretion lead to different functional properties. Ann. Rev. Immunol. 7:145-158.

Hori S, Nomura T, Sakaguchi S (2003). Control of regulatory T-cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

Sakaguchi S (2004). Naturally arising $CD4^+$ regulatory T-cells for immunologic self-tolerance and negative control of immune responses. Annu. Rev. Immunol. 22:531-562.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995). Immunologic self-tolerance maintained by activated T-cells expressing IL-2 receptor □-chains (CD25). Break-down of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. 155:1151-1164.

Fontenot JD, Gavin MA, Rudensky AY (2003). Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T-cells. Nat. Immunol. 4:330-336.

Fukaura H, Kent, S. C., Pietrusewicz, M. J., Khoury, S. J., Weiner, H. L., and Hafler, D. A. 1996. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-□1-secreting $T_H3$ T-cells by oral administration of myelin in multiple sclerosis patients. J. Clin. Invest. 98:70-77.

Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK (2001). Type 1 T-regulatory cells. Immunol. Rev. 182:68-79.

Kim JM, Rasmussen JP, Rudensky AY (2007). Regulatory T-cells prevent cata-strophic autoimmunity throughout the lifespan of mice. Nat. Immunol. 8:191-197.

O'Garra A, Vieira PL, Vieira P, Goldfeld AE (2004). IL-10-producing and naturally occurring $CD4^+$ $T_{regs}$ cells: Limiting collateral damage. J. Clin. Invest. 114:1372-1378.

Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H (2005). Inducing and expanding regulatory T-cell populations by foreign antigen. Nat. Immunol. 6:1219-1227.

Mucida D, Kutchukhidze N, Erazo A, Russo M, Lafaille JJ, Curotto de Lafaille MA (2005). Oral tolerance in the absence of naturally occurring $T_{reg}$ cells. J. Clin. Invest. 115:1923-1933.

Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK (2006). Reciprocal developmental pathways for the generation of pathogenic effector $T_H17$ and regulatory T-cells. Nature 441:235-238.

Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH (2001). Regulatory $CD4^+CD25^+$ T-cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res. 61:4766-4772.

Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H, Fu YX (2005). Intra-tumor depletion of CD4 cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. J. Exp. Med. 201:779-791.

Shimizu J, Yamazaki S, Sakaguchi S (1999). Induction of tumor immunity by removing $CD25^+CD4^+$ T-cells: A common basis between tumor immunity and autoimmunity. J. Immunol. 163:5211-5218.

Yamamoto M, Kamigaki T, Yamashita K, Hori Y, Hasegawa H, Kuroda D, Moriyama H, Nagata M, Ku Y, Kuroda Y (2009). Enhancement of anti-tumor immunity by high levels of $T_H1$ and $T_H17$ with a combination of dendritic cell fusion hybrids and regulatory T-cell depletion in pancreatic cancer. Oncol. Reports 22:337-433.