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ORIGINAL ARTICLE

Antigen Specific Immune Responses In Mice Subjected To Infrared Heat Stress

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Short exposures to infrared heat are generally used to facilitate tail-vein bleeding of experimental mice as an alternative to retro-orbital bleeding for the purpose of obtaining serum samples. Altered temperatures have been shown to influence immune responses in a variable manner. This study evaluates the effects of infrared heat on the immune response. After confirming the efficacy of heat exposure as measured by alterations in body temperatures, the exposed mice were evaluated for antigen-specific antibody responses and allogeneic cytotoxic T lymphocytic (CTL) responses as readouts for humoral and cellular immune responses respectively. Antigen-specific serum antibody titers to lysozyme, bovine serum albumin, ovalbumin, diphtheria toxin and rabbit IgG antigens were analyzed in infrared heat exposed and unexposed control C57BL/6 mice that were immunized with the corresponding antigen. Significant decreases in antigen-specific antibody titers were observed only when heat exposed C57BL/6 mice were immunized with lysozyme or BSA but not with other antigens tested. These alterations were not seen in heat exposed BALB/c mice. Dialyzed serum prepared from such heat exposed C57BL/6 was also found to inhibit CTL generation *in vitro* and inhibited IL-2 stimulated proliferation of CTLL-2 cells. These studies suggest that the procedure of infrared heat exposure prior to tail vein bleeding may influence some immune parameters although this may not be true for all strains of mice and all antigens.

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Key words: infrared heat, serum antibodies, CTL

Environmental stress such as heat stress is a stimulus that disturbs physiological equilibrium (Sareh et al., 2011; Salak-Johnson and McGlone, 2007; Harikai et al., 2003) and elevated temperature is known to alter immune responses (Wetsel, 2011; Morrow-Tesch et al., 1994; Yoshioka et al., 1990). Exposure of rats to heat stress resulted in suppressed DTH response to keyhole limpet haemocyanin, decreased number of peripheral blood lymphocytes, total T cells, T helper cells as well as thymus size. On the other hand, serum anti-tetanus toxoid IgG antibodies were enhanced while proliferative response of Con-A stimulated splenic lymphocytes remained unaltered (Chayoth et al., 1988). Acute hyperthermia (rectal temperature 42°C) enhanced lymphocyte functions, proliferative responses to

alloantigens, PHA and Con-A, while chronic hyperthermia was associated with suppressed T cell proliferative responses that returned to normal levels 40 days after induction of hyperthermia (Anderson and Kuhn., 1989). PHA and Con-A induced blastogenesis of PBL was considerably decreased in heat stressed sheep. Addition of serum from heat stressed sheep also significantly suppressed the blastogenesis of lymphocytes obtained from human, bovine and sheep donors suggesting that the suppressive effect was species non-specific. Susceptibility of animals to infectious agents has also been shown to alter under stressful conditions (Jin et al., 2011; Klein, 1993).

Experimental mice are routinely bled from the retro-orbital sinus or tail vein for the purpose of obtaining serum samples. Tail vein bleeding is preferred when damage to the eyes needs to be avoided. Short exposures to infrared heat have been used to enhance blood flow to facilitate tail vein bleeding by increasing body temperature and infrared heat treatment has been shown to alter food intake in rats (Hu et al., 2011; Joseph et al., 1991). We show in this report that lysozyme-specific antibody responses are lowered in C57BL/6 but not BALB/c mice that were subjected to infrared heat exposure prior to tail vein bleeding. We further show that this does not occur with all antigens suggesting that the effects of infrared heat exposure employed for tail vein bleeding may be strain and antigen-dependent.

MATERIALS AND METHODS

Reagents

Ovalbumin, electrophoresis reagents and RPMI 1640 were purchased from Sigma chemical company, USA. Minimum Essential Medium (MEM), gentamycin, streptomycin, penicillin, sodium pyruvate, L-glutamine, non essential amino acids (NEAA), Coomasie Brilliant Blue R 250 and Lysozyme were purchased from Hi-Media Pvt. Ltd., India. Fetal Bovine Serum (FBS) was from GIBCO, USA. Tissue culture ware was purchased from Nunc, Netherlands. Cellulose Acetate membranes used for sterile filtration of media were purchased from Millipore, India and Advanced microdevices, India. $Na_2{}^{51}CrO_4$ (specific activity usually ranging between 65-175 μCi/mg in different batches) and Tritiated thymidine (specific activity 6,500 mCi/mMole) were purchased from Board of Radiation Isotope Technology (BRIT), India.

Cells and Media

P815 (H-2^d), a DBA/2 derived mastocytoma and EL-4 (H-2^b), a C57BL/6 mouse derived T cell lymphoma were obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. All cultures were done at 37°C in a 5% CO_2 incubator.

Media used routinely to maintain cell lines was RPMI 1640 supplemented with antibiotics (100 units/ml penicillin, 250 μg/ml streptomycin and 50 μg/ml gentamycin), L-glutamine (0.32 mg/ml) and 5% FBS. Mixed lymphocyte culture (MLC) medium containing RPMI 1640 supplemented with sodium pyruvate (0.12 mg/ml), non essential amino acids (0.1 mg/ml), 2-mercaptoethanol (5 x 10^{-4}M) and 5% FBS was used for cytotoxic T lymphocyte (CTL) cultures. CTLL-2 cells were routinely grown in RPMI 1640 medium that was supplemented with 5% FBS and exogenously added 20U/ml recombinant IL-2.

Antibodies

Biotinylated Goat anti-mouse IgG (whole molecule), Rabbit anti-mouse IgG (whole molecule), Avidin peroxidase, Rabbit anti-mouse IgG (whole molecule) peroxidase conjugate were purchased from Bangalore Genei, Bangalore.

Mice and blood collection

Inbred strains of C57BL/6 and BALB/c mice were obtained from the Central animal facility, Indian Institute of Science and immunized subcutaneously (s.c.) or intraperitoneally (i.p.) with antigen (20µg/mouse) after emulsification with crude Freund's adjuvant (CFA) on day 0 and 21 unless otherwise stated. Sera were collected from control and exposed mice on day 0, 14, 21, 28 and 35. Heat exposure of mice was carried out on day 1, 15, 22 and 29. All mice were subjected to light ether anesthesia prior to retro-orbital bleeding. For testing in CTL cultures, serum was obtained by tail vein bleeding immediately or after different times of heat exposure from an infrared source for 4 min as given below.

Heat Exposure

Mice were enclosed in a 500 ml glass beaker and exposed to heat for a period of 4 min. The heat was generated from an infrared lamp. Each mouse was positioned in the inverted beaker such that the tail protruded through the spout of the beaker. The tail was held tight not only to curtail panic-induced sudden movements, but also to ensure direct frontal exposure of the animal. Control animals were handled similarly using a fresh glass beaker but the infrared lamp was switched off.

Measurement of internal body temperature in mice

Rectal temperature was measured with a digital thermometer (LDC portable digital multistem thermometer with external sensing probe, Singapore) every two min over a period of 15 min. The temperature measured before initiation of stress was taken as the normal body temperature. The average of three readings was taken for each measurement using three mice per group.

ELISA

Antibody titres in sera of mice were determined by Avidin-Biotin micro ELISA. All assays were carried out in 96 well flat bottomed polystyrene plates (Costar, USA). 0.1 ml of antigen at a concentration of 5 µg/ml in 0.1M sodium carbonate-bicarbonate buffer, pH 9.6, was coated onto each well and the plate was incubated for 30 min at 37°C. After incubation, excess antigen was removed from the well by washing with rinse buffer (PBS with 0.1% tween 20). Non-specific sites were blocked by addition of 0.3 ml serum diluent buffer (SDB, containing PBS with 0.1% tween-20 and 5% goat serum) into each well and incubated for 1 hour 15 min at 37°C. After washing, 0.1 ml of appropriately diluted antiserum was added to each well and the plate was incubated for 30 min at 37°C. Excess antiserum was removed by washing the wells and 0.1 ml of 1:1000 diluted secondary antibody (goat anti-mouse IgG biotinylated) was added to each well and the plate was incubated for 30 min at 37°C. Wells were washed and 0.1 ml of 1:1000 diluted Avidin-HRP conjugate was added to each well was incubated for 8 min at 37°C. After incubation, wells were washed and 0.1 ml of substrate (0.5 mg OPD/ml PO_4 buffer 0.2 M, pH 7.0/1 µl of 6% H_2O_2) was added to each well and the plate was incubated for 25 min at room temperature. The reaction was terminated by addition of 0.1 ml of 2N HCl and the color developed was read at 490 nm in a microplate autoreader (Bio-Tek instrument) ELISA Reader.

Centricon dialysis and concentration

Serum obtained from control and heat exposed mice were subjected to centrifugal concentration and dialysis on 30 kDa Centricon membrane filters (Amicon Inc, Beverly, MA 01915, USA). Briefly, 0.2 ml of serum along with 0.4 ml MLC medium

(without FBS) was subjected to Centricon centrifugal concentration at 6000 rpm for 1 hr in a Kubota centrifuge (Model Kubota KR-20000 T). After centrifugation, 0.4 ml of medium was added to the serum component retained above the membrane filter (retentate) and centrifuged for a further period of 1 hr. This step was repeated once again. The filtrate passing through the filter was collected and stored separately in a tissue culture tube at -20°C until use. The retentate cup was reverse spun at 2000 rpm for 2 min to collect the retained protein fraction. 0.6 ml of medium was added to wash the top of the membrane filter and reverse spun again to collect the washings. Retentate thus obtained contained serum components that were more than 30 kDa and was stored at -20°C until further use. Both retentate and filtrate fractions (less than 30 kDa) were tested at various concentrations that were expressed as the final serum equivalents from which they were prepared.

Allogeneic Cytotoxic T Lymphocyte (CTL) generation and assay

C57BL/6 (6-8 weeks old) mice were primed *in vivo* by immunizing them with $1x10^7$ BALB/c splenocytes intraperitoneally (i.p) on day 0. Animals were sacrificed after a 12 day period and an *in vitro* allogeneic CTL culture was set up by the co-culture of $5x10^6$ responder C57BL/6 splenocytes with $2x10^6$ irradiated BALB/c splenocytes in 2 ml MLC culture medium per well in 24-well Linbro plates for 5 days at 37° C in a CO_2 incubator.

Effector cells generated during the 5 day culture period were assayed for their cytotoxic activity in a 4-5 hr Chromium release assay. Varying number of effector cells were incubated with $2x10^4$ target cells (P815, EL-4) in a 96 well V bottomed plate for 4 hr at 37°C and 5% CO_2 atmosphere after spinning the plates at 400 rpm for 4 min. Target cells were prepared by preincubating cells with 100 µCi of $Na_2{}^{51}CrO_4$ for 1 hr at 37°C. Excess chromium was removed by washing the cells thrice. At the end of the incubation period plates were spun down at 1200 rpm for 5 min and 0.1ml of cell free supernatant was counted in a LKB Minigamma counter. Percent lysis was calculated using the formula: (cpm released in the presence of effectors minus spontaneous release/total cpm released with detergent minus spontaneous release) x 100.

CTLL-2 Proliferation assay

The effect of serum filtrate and retentate obtained from control and heat exposed C57BL/6 was tested on IL-2 induced proliferation of CTLL-2 cells (a cloned murine cytotoxic T cell line). CTLL-2 cells cultured in IL-2 medium were washed twice with medium to remove residual IL-2 and added to 96 well plates. Cells ($1x10^4$/well) in MLC medium containing 0.2 U/ml IL-2 and appropriately diluted samples to be tested were cultured at 37°C and 5% CO_2 atmosphere in a 96 well flat bottomed tissue culture plate. Control well contained cells only. Two hours before harvesting, the cultures were pulsed with $4x10^5$ ($[^3H]$-thymidine) cpm per well. At the end of incubation period, cells were harvested onto glass fiber filters using Nunc semi automated cell harvester. Samples were counted in a LKB Rack Beta counter using scintillation fluid (0.5% PPO and 0.025% POPOP in toluene). The standard proliferation curve obtained from the 36 hrs culture with added IL-2 was linear over the range of 1.5 units to 14 units yielding 5000 to 10,000 cpm. Data represent mean ± SD of IL-2 U/ml of culture supernatants.

Table 1: Anti-lysozyme response in C57BL/6 mice

DAY OF BLEED[a]	ELISA OD 490 nm	
	TAIL VEIN[b]	RETRO-ORBITAL[c]
28	0.25 ± 0.08 (0.29, 0.35, 0.10)	1.87 ± 0.36 (2.3, 1.15, 2.16)
35	0.21 ± 0.03 (0.22, 0.16, 0.25)	2.07 ± 0.18 (2.29, 1.72, 2.19)

[a]Lysozyme (10µg/mouse) immunization (i.p.) was done with aluminium hydroxide (5mg) on day 0 and day 21.
[b]Mice were bled after exposure to infrared heat for 4 min and tested at 1:500 serum dilution.
[c]Mice were bled under light ether anesthesia and tested at 1:500 serum dilution.

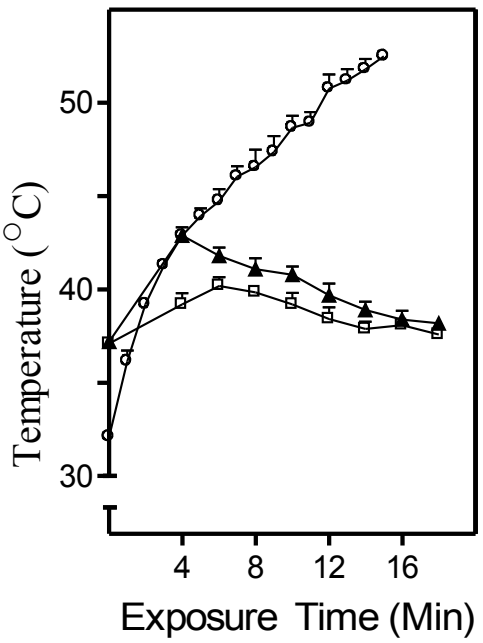

Figure 1. Measurement of body temperature. C57BL/6 (▲) or BALB/c (□) mice were subjected to infrared lamp heat exposure for 4 min as described in *Materials and Methods*. Rectal temperature for both strains of mice was recorded thereafter at regular intervals over a period of 14 min after cessation of exposure. Temperature inside the empty inverted glass beaker (o) was also recorded for a period of 16 min with the thermometer inside the empty beaker while the infrared lamp was switched on continuously. Data is represented as mean rectal temperature recorded at each time interval ± SD for 3 mice per group.

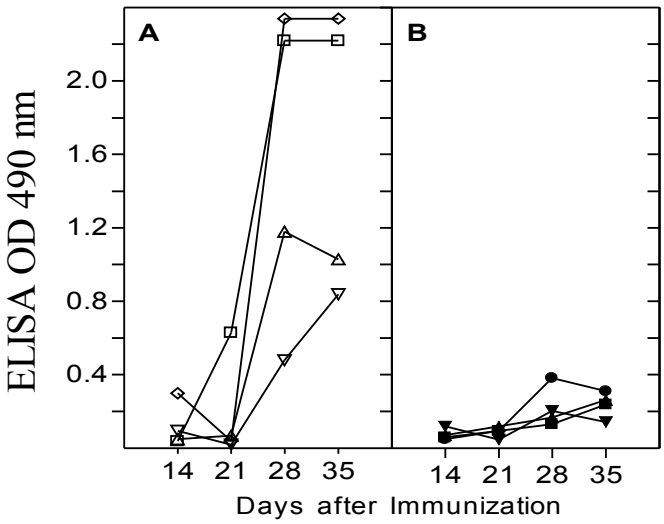

Figure 2. Lysozyme-specific antibody response in C57Bl/6 mice. Mice immunized (i.p) with lysozyme on day 0 and 21 were subjected to heat exposure on day 1, 15, 22 and 29. Anti lysozyme IgG antibody titers were measured in serum of control (A) and heat exposed (B) mice that were obtained on day 14, 21, 28 and 35 as given in *Materials and Methods* section. ELISA was performed at several antiserum dilutions but absorbance values at 490 nm are given only for one representative antiserum dilution (1:500). Each curve represents the values obtained for individual mice at different time points. All mice were bled retro-orbitally.

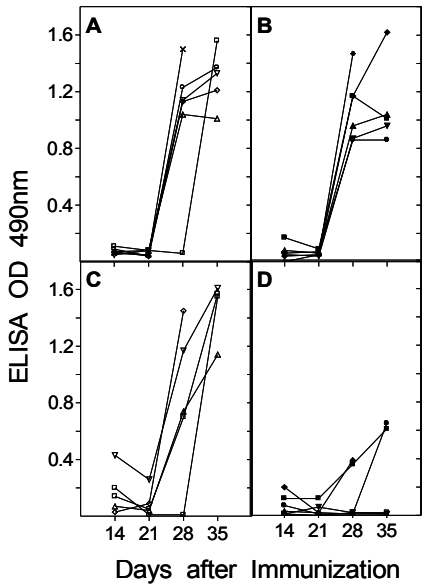

Figure 3. Antibody responses to BSA in heat exposed mice. BALB/c (A and B) and C57BL/6 (C and D) mice were immunized (s.c.) on day 0 and 21 with BSA and subjected to heat exposure on day 1, 15, 22 and 29. Anti BSA IgG antibody titers were measured in serum of control (Panels A and C) and heat exposed (Panels B and D) mice that were obtained on day 14, 21, 28 and 35 as given in *Materials and Methods* section. ELISA was performed at several antiserum dilutions but the absorbance values at 490 nm are given only for one representative antiserum dilution (1:20,000 for C57BL/6 and BALB/c mice). Each curve represents the values obtained for individual mice at different time points. All mice were bled retro-orbitally. Panel A: control BALB/c mice; Panel B: heat exposed BALB/c mice; Panel C: control C57BL/6 mice; Panel D: heat exposed C57BL/6 mice.

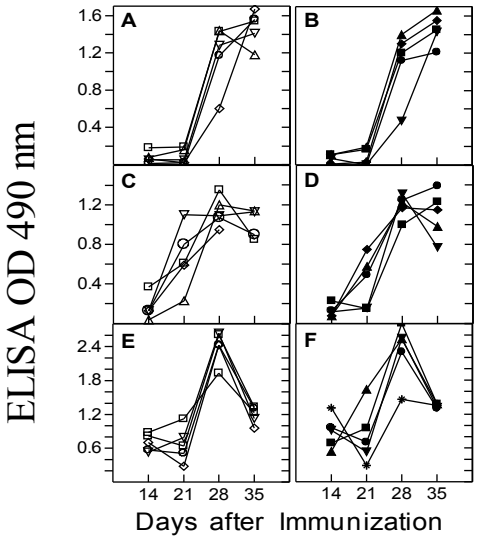

Figure 4. Antibody response in heat exposed C57BL/6 mice. C57BL/6 mice were immunized (s.c) on day 0 and 21 with ovalbumin, diphtheria toxin and rabbit IgG. Antigen-specific antibody titers were measured by standard Avidin-Biotin ELISA in the serum of control (Panels A, C, E) and heat exposed (Panels B, D, F) mice on day 7, 14, 21, 28 and 35. Heat exposure was carried out on day 1, 15, 22 and 29. Antibody titers were determined at several antiserum dilutions but the values obtained for one representative antiserum dilution (1:400 in A and B; 1:2000 in C and D; 1:10000 in E and F)are plotted. Each curve represents the values obtained for individual mice at different time points. All mice were bled retro-orbitally. Panels A and B: Anti ovalbumin response in control and heat exposed mice; Panels C and D: Anti diphtheria toxin response in control and heat exposed mice; Panels E and F: Anti rabbit IgG response in control and heat exposed mice.

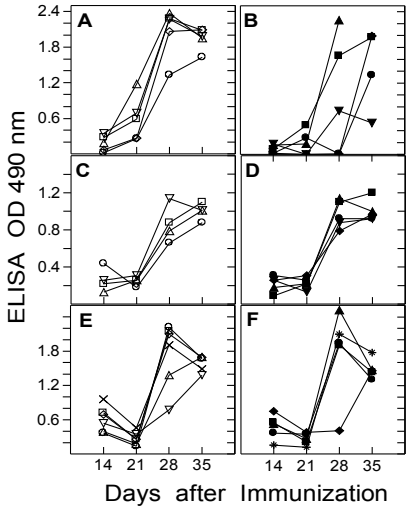

Figure 5. Antibody response in heat exposed BALB/c mice. BALB/c mice were immunized (s.c) on day 0 and 21 with ovalbumin, diphtheria toxin and rabbit IgG. Antigen-specific antibody titers were measured by standard Avidin-Biotin ELISA in the serum of control (Panels A, C, E) and heat exposed (Panels B, D, F) on day 7, 14, 21, 28 and 35. Heat exposure was carried out on day 1, 15, 22 and 29. Antibody titers were determined at several antiserum dilutions but the values obtained for one representative antiserum dilution (1:2000 in A and B; 1:2000 in C and D; 1:10000 in E and F) are plotted. Each curve represents the values obtained for individual mice at different time points. All mice were bled retro-orbitally. Panels A and B: Anti ovalbumin response in control and heat exposed mice; Panels C and D: Anti diphtheria toxin response in control and heat exposed mice; Panels E and F: Anti rabbit IgG response in control and heat exposed mice.

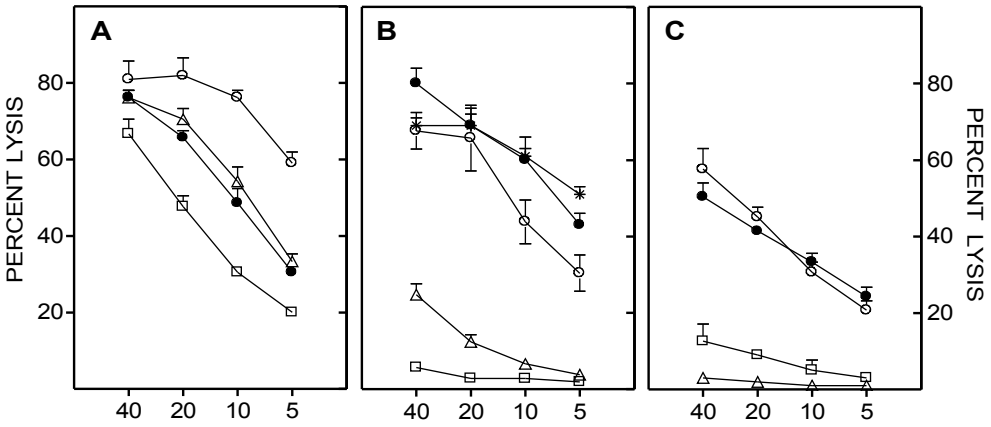

Figure 6. Effect of centricon dialyzed serum from heat exposed mice on CTL generation. Spleen cells from C57BL/6 mice that were primed with BALB/c splenocytes 12 days earlier were restimulated *in vitro* with irradiated BALB/c splenocytes in the presence of medium alone (○), medium supplemented with 1.5% (●), 3% (Δ), 4.5% (□) equivalent serum retentate or 4.5% equivalent serum filtrate (∗) prepared from normal (Panel A) or heat exposed (Panel B) C57BL/6 mouse serum. Panel C represents the lysis obtained when the retentate added to the culture was prepared from serum obtained at 0 (Δ), 6 (□) or 12 (●) hr after heat exposure of C57BL/6 mice. Effector cells obtained after 5 day culture period were assayed on P815 targets in a 5 hr chromium release assay. Data is represented as percent lysis obtained at four different E:T (Effector:Target) ratios ± SD.

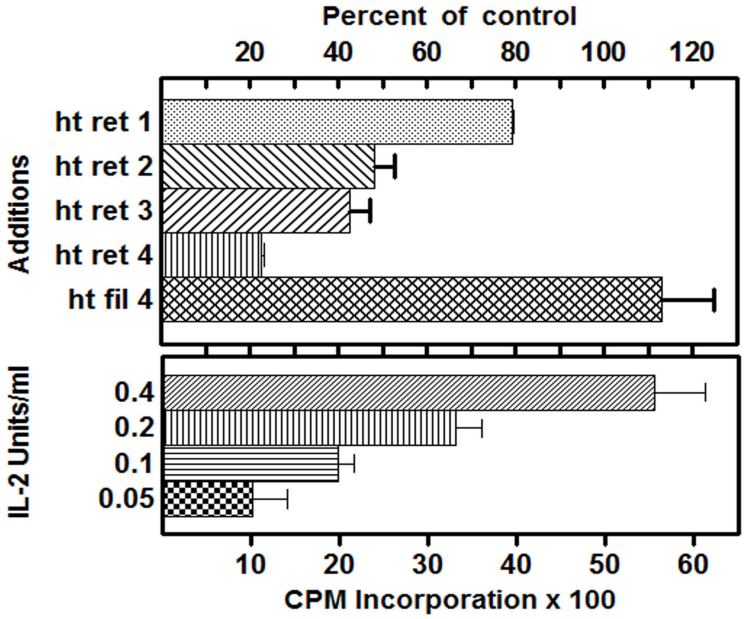

Figure 7. Effect of centricon dialyzed serum on CTLL-2 proliferation. Top panel: CTLL-2 cells were cultured in medium containing 0.2 U/ml IL-2, either in the absence (control) or presence of retentate or filtrate prepared from serum of heat exposed mice. Histograms marked ht ret 1, 2, 3 and 4 represent the addition of 1x, 2x, 3x and 4x concentrations of retentate where 4x was equivalent to 9% serum and ht fil 4 represents the corresponding filtrate control. Bottom panel: Dose response of IL-2 on CTLL-2 cell proliferation. CTLL-2 cells were cultured in the presence of medium supplemented with increasing concentrations of IL-2 as given. All cells were pulsed with ^{3}[H] thymidine and harvested as described in *Materials and Methods* section. All assays were performed in triplicates and results are expressed as percent of control response.

RESULTS

Internal body temperature

Rectal body temperatures were initially evaluated to confirm the alteration of normal physiological equilibrium in the animal upon exposure to stress. Towards this, the first study conducted was the measurement of body temperature. C57BL/6 and BALB/c mice were separately subjected to heat exposure as described in *Materials and Methods* section and rectal temperatures were recorded as an index of internal body temperature.

Temperature was recorded over a period of 20 min in both strains of mice. Data is represented as mean temperature ± SD recorded for 3 mice in each group. Prior to exposure of mice, temperature changes inside the glass beaker were first recorded by placing the thermometer inside the empty inverted glass beaker while the infrared light was switched on. There was a gradual rise in temperature inside the beaker with time. Ambient temperature recorded before switching on the infrared lamp was 32 ± 0.8°C. As shown in **Fig 1**, there was a rise of 10.7°C at the end of the 4 min exposure. Temperature was found to rise continuously in the beaker if the light was left on. The period of exposure was confined to 4 min only to avoid death. Temperature recorded in heat exposed C57BL/6 mice indicated that there was a rise in the normal body temperature from 37.5°C to 42.7°C after 4 min heat exposure. The rise in temperature inside the beaker correlated with the temperature rise recorded for C57BL/6 mice that were subjected to stress during the 4 min period of infrared heat exposure as shown in **Fig 1**. Heat exposure of BALB/c mice led to a rise in body temperature from a normal of 36.9°C to 40°C after 4 min heat exposure as shown in **Fig 1**. It was also observed that the rise in body temperature of C57BL/6 was higher than that of BALB/c mice. During cold stress studies that involved exposure of mice to -20°C for 20 min, the body temperature dropped from a normal of 36.4°C to 27.5°C in C57BL/6 mice and from 36.3°C normal to 25.3°C after cold exposure in BALB/c mice (Supplementary Fig 1). The body temperature in either heat or cold exposed mice gradually returned to normal over a period of 16–20 min after cessation of stress.

Antigen-specific antibody titers in heat exposed mice

Bleeding by tail vein nicking involves exposure of mice to infrared heat in order to enhance blood flow to the tail by increasing body temperature. The rise in normal body temperature in heat exposed mice as shown in **Fig 1** was indicative of hyperthermic conditions and elevation in rectal temperature (rectal temperature 42ºC) has been reported to cause alterations in the immune functions in mice (Solov'ev., 1992). Since tail-vein bleeding generally involves a facilitating exposure to infrared heat, we compared the antibody titers induced by lysozyme immunization in animals that were bled through the tail vein or the retroorbital plexus. As shown in **table 1**, lysozyme-specifc serum antibody titers in lysozyme immunized C57BL/6 mice were significantly lower when bled by tail vein nicking that involved brief exposure to infrared heat when compared to retro-orbital bleeding that did not involve heat exposure. The time course of antibody responses in mice that were immunized with lysozyme in the presence of aluminum hydroxide as adjuvant is shown in Fig 2. Antibody titers to lysozyme in heat exposed C57BL/6 mice were lower on day 28 and 35 while the titers remained unaltered on day 14 and 21 post lysozyme immunization as shown in **Fig**.**2**.

C57Bl/6 mice are categorized as low responders to lysozyme (Sadegh-Nasseri et al., 1984; Riley et al., 1982) and lysozyme immunization involved aluminium hydroxide as the adjuvant. Hence it was of interest to analyze the antigen-specific antibodies to other antigens in the presence of CFA as the adjuvant for stronger stimulation. Serum antibody titers to BSA obtained at 1:20,000 dilution were low on days 14 and 21 following primary immunization as shown in **Fig 3.** However, the ELISA OD values obtained at 1:20,000 serum dilution on day 28 and 35 bleeds increased following secondary immunization. Heat exposure had no significant effect on the anti-BSA titers in BALB/c mice on all the days tested (**Fig 3A&B).** On the other hand, the increase in OD values seen in heat exposed C57BL/6 mice was much lower than unexposed controls (**Fig 3E&D**).

These observations prompted us to analyze the effect of infrared heat exposure on the circulating antibody levels to ovalbumin, diphtheria toxin, and rabbit IgG in C57BL/6 and BALB/c mice. In contrast, to anti-BSA titers, no significant changes were observed in heat exposed BALB/c (**Fig 4**) and C57BL/6 (**Fig 5**) mice in the case of other antigens such as ovalbumin (Panels A&B), diphtheria toxin (Panels C&D) or rabbit IgG (Panels E&F) either in the case of BALB/c or C57BL/6 mice. This suggested that the effect of heat exposure on antigen-specific responses may not be observed in all mouse strains or for all antigens.

Effect of heat serum retentate and filtrate on allogeneic CTL generation

Many stress mediated immunosuppressive effects have been ascribed to elevated levels of corticosteroids which is characteristic of acute stress responses (Sapolsky and Donnelly., 1985). Other factors that may play an important role include catecholamines (Fujiwara and Orita., 1987) as well as endogenous opiates (Shavit et al., 1986). Also acute heat exposure has been shown to cause rat pituitary corticotroph activation (Jasnic et al., 2010). In order to determine if the suppressive effects of heat exposure was due to such factors/hormones of low molecular weight, serum obtained from heat stressed mice was concentrated on 30 kDa Centricon membrane filters as already described. The dialysed filtrate and retentate thus obtained were supplemented in MLC medium to analyze their effects on allogeneic CTL generation. In order to determine the dose-dependency of the inhibition, allogeneic CTL cultures (C57BL/6 anti BALB/c (**Figure 6)** were set up with different doses (1.5-4.5% serum equivalent) of retentate prepared from unexposed (control retentate) serum (**panel A**) or heat exposed (heat retentate) serum (**panels B and C**) C57BL/6 mice. Data is represented as percent lysis obtained at different E:T ratios. As shown, inhibition caused by the filtrate was marginal (11% at E:T 40:1) relative to the retentate which exhibited significant inhibition of CTL generation *in vitro*. The dose dependence of the inhibition is shown in **panel B**. Significant inhibition of CTL generation was observed with a dose of 3% (24.8% vs 80% lysis at E:T, 40:1) and 4.5% serum equivalent retentate (5.7% vs 80% lysis at E:T, 40:1) inhibited allogeneic CTL generation. Time course experiments indicated that the inhibitory activity was present in serum for at least 6 hr (**panel C**) after heat exposure. These experiments suggested that the inhibitory factor in the serum was of molecular weight higher than 30 kDa and that the inhibition may not be due to higher circulating concentration of immunosuppressive steroids released upon heat exposure.

Effect of retentate on proliferation of mouse CTLL-2 cells

T cell activation due to antigen binding and

costimulation renders the antigen activated T cell sensitive to the proliferative signal usually provided by T cell-derived IL-2 for CTL precursors. Reports indicate that soluble mediators derived from a variety of sources act either to limit IL-2 production or to neutralize IL-2 activity (Gautam et al., 1983; Hardt et al., 1981). Normal mouse serum was shown to contain a circulating inhibitor which neutralizes IL-2 activity (Honda et al., 1985). To test out these possibilities, retentate and filtrate prepared from serum of unexposed and heat exposed C57BL/6 mice were examined for their inhibitory effects on the proliferation of murine IL-2 dependent cytotoxic T cells (CTLL-2) as measured by [^{3}H]-thymidine uptake. Retentate and filtrate preparations used in the experiments were obtained as described earlier. The linearity of IL-2 induced proliferation with increasing concentrations of IL-2 is shown in **Fig 7 (Bottom)**. For further studies, the effect of retentate and filtrate additions were analyzed on the proliferation of CTLL-2 induced at a dose of 0.2 U/ml IL-2. Values obtained for cultures with 0.2 U/ml IL-2 without added retentate was taken as 100% and results are expressed as percent of control response. As shown in **Fig 7 (Top),** inhibition of CTLL-2 proliferation obtained with retentate prepared from serum of heat exposed C57BL/6 mice was dose dependant while the filtrate did not show any inhibition at the highest dose tested. The inhibition obtained with heat exposed retentate was higher (77%) than the retentate prepared from serum of unexposed (38.1%) mice (Supplementary Fig 2). Interestingly, filtrate prepared from either the serum of heat exposed (113.3%) or unexposed (113.5%) C57BL/6 mice showed modest stimulatory effects. These results suggested that the inhibitory effects of retentate addition may be mediated by inhibition of IL-2 action. Further experiments to determine if addition of excess exogenous IL-2 would cause reversal of the inhibition caused by retentate may answer the mode of retentate action on CTLL-2 proliferation.

DISCUSSION

Studies initiated on the effect of heat exposure on allogeneic CTL generation was based on our accidental observation that serum antibody titers to lysozyme were lower (**Table 1**) when bled by tail vein nicking compared to mice that were bled through retro-orbital plexus. Bleeding by tail vein nicking involves heat exposure of mice to an infrared lamp to enhance blood flow to the tail by increasing body temperature. Many studies support the association of immune suppression with stressful conditions. Heat exposure in animals has been shown to result in immunosuppression and tumor enhancement (Regnier and Kelley., 1981). It has also been shown that stressful conditions in animals lead to increased susceptibility to infectious diseases (Kelley, 1980; Klein, 1993).

Therefore, we have evaluated the effect of acute heat exposure caused by infrared lamp on mouse immune responses. Measurement of body temperature was used to evaluate the efficacy of infrared heat exposure (**Fig 1**). Body temperature of both C57BL/6 and BALB/c mice increased after 4 min of infrared light exposure. Similar elevations in body temperature of mice exposed to 42°C have been reported earlier (Solov'ev., 1992). The rise in body temperature returned to normal levels over a period of 16 min after cessation of stress. As controls, neither cold nor immobilization stress caused any rise in body temperature in both strains of mice (Supplementary Fig 1).

It has been reported that acute hyperthermia (rectal temperature 42°C) caused enhanced lymphocyte function, proliferative responses to alloantigens and mitogens such as PHA and Con A

(Solov'ev., 1992). On the other hand, mice subjected to chronic hyperthermia showed suppressed T cell response while local hyperthermia (Yoshioka et al., 1990) caused a biphasic increase in NK cell activity (Cheng et al., 1990).

The literature on the effect of heat exposure on CTL generation is scanty. It has been shown that exposure of mice to cold water stress caused reduced NK cell activity (Cheng et al., 1990). Immobilization stress in rats (Steplewski and Vogell., 1986) increased the numbers of neutrophils and large granulocytes while the numbers of helper and suppressor T cells were significantly decreased. Based on these observations, these authors have suggested that stress can differentially influence individual immune compartments of the cellular immune system in a selective manner. In our studies, heat stress significantly suppressed antibody responses.

Heat exposure had no effect on the antibody titers to ovalbumin, diphtheria toxin and rabbit IgG both in C57BL/6 and BALB/c mice (**Fig 4 and 5**). However, antibody responses to lysozyme (**Fig 2**) and BSA (**Fig 3**) were lower only in C57BL/6 but not in BALB/c mice. The reason for this observation is as yet unclear but could possibly be due to differential stress-hormone induced responses of the lymphocytes in stressed C57BL/6 mice relative to stressed BALB/c. The importance of stress factors in immune responses is also supported by the observation that susceptibility and resistance to EAE in Lewis and PVG rats was related to the strain-dependent release of corticosterone from the adrenal glands. PVG rats were resistant to EAE induction and had higher levels of serum corticosterone than Lewis rats (Levine et al., 1980; Mason et al., 1990). Also, the rise in body temperature of heat exposed C57BL/6 mice was relatively higher than BALB/c mice **(Fig 1)**. Hence further experiments on CTL generation were performed in C57BL/6 mice.

Several reports (Joseph et al., 1991; Dhabhar et al., 1996) indicate that stress alters the number of lymphocytes in different immune compartments but the recovery of splenocytes in heat exposed and control animals did not decrease significantly in our studies. Hence altered antibody responses in heat exposed C57BL/6 mice could not be explained by losses in recovery of splenocytes. Similarly it has been observed that the absolute numbers of T and B cells remained unaltered although the mitogenic response of T lymphocytes was suppressed in stressful conditions such as bereavement (Tecoma and Huey., 1985).

It has been reported (Niwano et al., 1990) that serum from heat stressed sheep suppressed PHA induced proliferation of lymphocytes. Also, the generation of allogeneic CTL culture was lower in heat exposed C57BL/6 mice but the decrease was modest and variable (data not shown) possibly due to the effects of *in vitro* stimulation. Hence, in order to identify the presence of suppressive effects, serum from heat exposed C57BL/6 and BALB/c mice or serum from unexposed C57BL/6 mice were evaluated for their inhibitory effects on allogeneic CTL generation. In order to avoid the presence of low molecular weight suppressive factors such as corticosteroids and to avoid non-specific effects, serum obtained from heat exposed mice was subjected to dialysis and concentration on 30 kDa Centricon membrane filters to remove low molecular weight substances and peptides. While CTL cultures generated in the presence of serum filtrate remained unaffected and in fact, showed modest stimulation, the addition of 3% equivalent or more of serum retentate inhibited C57BL/6 anti BALB/c CTL generation significantly. Our time

course experiments indicate that the retentate was present in circulation for 6 hr (**Fig 6B and 6C**).

It has been shown that the non-specific serum inhibitors present in the serum can suppress immune response either by directly inhibiting the action of IL-2 or by inhibiting IL-2 production by immune cells (Miossec et al., 1990; Lelchuk et al., 1985). We also observed that retentate prepared from serum of heat exposed C57BL/6 mice inhibited IL-2 induced proliferation of CTLL-2 cells to a higher extent than retentate from serum of unexposed mice (**Fig 7**). This also supported the observation that modest inhibition of CTL cultures was also observed with 4.5% equivalent of unexposed control serum **(Fig 6A)**. It was clear from the above studies that the inhibitory component/s was not a low molecular weight substance since the activity was retained on 30 kDa Centricon membrane filters and that it was present in normal animals but its concentration increases upon infrared heat exposure.

The inhibition of antigen-specific antibody responses to BSA and lysozyme were more apparent in C57BL/6 mice and not in BALB/c mice. Similarly, serum retentate was more effective in inhibiting CTL generation when obtained from C57BL/6 mice (not shown). The reason for this is yet unclear but the non-responsiveness of BALB/c mice could arise due to differences in the response of individual strains to stressors. Similar observation has been reported by Sei (Sei et al., 1992). It was observed that AKR/J mice subjected to periodic exposure to death of cage cohorts resulted in decreased allogeneic CTL generation. However, the response of C3H/HeJ and BALB/c mice was different suggesting the possibility of strain differences in the manner of response to a stressor.

Although thermal stress-induced suppressive effects on immune responses have been reported, the mechanisms underlying heat stress-mediated immune suppression still remains unclear (Niwano et al., 1990) and this report is important since it shows that infra-red exposure of mice, a procedure that is used to facilitate tail bleeding could differentially alter immune responses depending on the antigen and the mouse strain.

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REFERENCES

Anderson, K.J. and Kuhn, R.E. (1989). Elevated environmental temperature enhances immunity in experimental Chagas' disease. *Infect Immun.,* **57,** 13-7.

Chayoth, R., Christou, N.V., Nohr, C.W., Yale, J.F., Poussier, P., Grose, M., Montambault, M., Chan, W. and Marliss, E.B. (1988). Immunological responses to chronic heat exposure and food restriction in rats. *Am J Clin Nutr.,* **48**, 361-7.

Cheng, G.J., Morrow-Tesch, J.L., Beller, D.I., Levy, E.M. and Black, P.H. (1990). Immunosuppression in mice induced by cold water stress. *Brain Behav Immun*., **4**, 278-91.

Dhabhar, F.S., Miller, A.H., McEwen, B.S. and Spencer, R.L. (1996). Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones. *J Immunol*., **157,** 1638-44.

Fujiwara, R. and Orita, K. (1987). The enhancement of the immune response by pain stimulation in mice. I. The enhancement effect on PFC production via sympathetic nervous system in vivo and in vitro. *J Immunol.,* **138**, 3699-703.

Gautam, S.C., Hilfiker, M.L. and Battisto, J.R. (1983). In vivo development of cytolytic T

lymphocytes (CTL) to hapten-altered self: MIs-disparate cells facilitate the response by neutralizing IL 2 inhibitor. *J Immunol*., **130**, 533-7.

Harikai, N., Tomogane, K., Miyamoto, M., Shimada, K., Onodera, S. and Tashiro, S. (2003). Dynamic responses to acute heat stress between 34 degrees C and 38.5 degrees C, and characteristics of heat stress response in mice. *Biol Pharm Bull*., **26**, 701-8.

Hardt, C., Rцllinghoff, M., Pfizenmaier, K., Mosmann, H. and Wagner, H. (1981). Lyt-23+ cyclophosphamide-sensitive T cells regulate the activity of an interleukin 2 inhibitor in vivo. *J Exp Med.,* **154**, 262-74.

Honda, M., Chan, C. and Shevach, E.M. (1985). Characterization and partial purification of a specific interleukin 2 inhibitor. *J Immunol.,* **135**, 1834-9.

Hu, J., Choo, H.J. and Ma, S.X. (2011). Infrared heat treatment reduces food intake and modifies expressions of TRPV3-POMC in the dorsal medulla of obesity prone rats. *Int J Hyperthermia*., **27**, 708-16.

Jasnic, N., Korac, A., Velickovic, K., Golic, I., Djordjevic, J., Djurasevic, S., Djordjevic, I., Vujovic, P. and Cvijic, G. (2010). The effect of acute heat exposure on rat pituitary corticotroph activation: the role of vasopressin. *Folia Histochem Cytobiol*., **48**, 507-12.

Jin, Y., Hu, Y., Han, D. and Wang, M. (2011). Chronic heat stress weakened the innate immunity and increased the virulence of highly pathogenic avian influenza virus H5N1 in mice. *J Biomed Biotechnol.,* 2011:367846. Epub 2011 May 29.

Joseph, I.M., Suthanthirarajan, N. and Namasivayam, A. (1991). Effect of acute heat stress on certain immunological parameters in albino rats. *Indian J Physiol Pharmacol*., **35**, 269-71.

Kelley, K.W. (1980). Stress and immune function: a bibliographic review. *Ann Rech Vet.,* **11**, 445-78.

Klein, T.W. (1993). Stress and infections. *J Fla Med Assoc.,* **80**, 409-11.

Lelchuk, R. and Playfair, J.H. (1985). Serum IL-2 inhibitor in mice. I. Increase during infection. *Immunology*., **56**, 113-8.

Levine. S., Sowinski. R. and Steinetz. B. (1980). Effects of experimental allergic encephalomyelitis on thymus and adrenal: relation to remission and relapse. *Proc Soc Exp Biol Med.,* **165**, 218-24.

Mason, D., MacPhee, I. and Antoni, F. (1990). The role of the neuroendocrine system in determining genetic susceptibility to experimental allergic encephalomyelitis in the rat. *Immunology*., **70**, 1-5.

Miossec, P., Elhamiani, M., Chichehian, B., D'Angeac, A.D., Sany, J. and Hirn, M. (1990) Interleukin 2 (IL 2) inhibitor in rheumatoid synovial fluid: correlation with prognosis and soluble IL 2 receptor levels. *J Clin Immunol*., **10**, 115-20.

Morrow-Tesch, J.L., McGlone, J.J. and Salak-Johnson, J.L. (1994). Heat and social stress effects on pig immune measures. *J Anim Sci*., **72**, 2599-609.

Niwano, Y., Becker, B.A., Mitra, R., Caldwell, C.W., Abdalla, E.B. and Johnson, H.D. (1990). Suppressed peripheral blood lymphocyte blastogenesis in pre- and postpartal sheep by chronic heat-stress, and suppressive property of

heat-stressed sheep serum on lymphocytes. *Dev Comp Immunol*., **14**, 139-49.

Regnier, J.A. and Kelley, K.W. (1981). Heat- and cold-stress suppresses in vivo and in vitro cellular immune responses of chickens. *Am J Vet Res*., **42**, 294-9.

Riley, R.L., Wilson, L.D., Germain, R.N. and Benjamin, D.C. (1982). Immune responses to complex protein antigens I. MHC control of immune responses to bovine albumin. *J Immunol*., **129**, 1553-8.

Sadegh-Nasseri, S., Kipp, D.E., Taylor, B.A., Miller, A. and Sercarz, E. (1984). Selective reversal of H-2 linked genetic unresponsiveness to lysozymes. I. Non-H-2 gene(s) closely linked to the Ir-2 locus on chromosome 2 permit(s) an antilysozyme response in H-2b mice. *Immunogenetics*., **20**, 535-46.

Sapolsky, R.M. and Donnelly, T.M. (1985). Vulnerability to stress-induced tumor growth increases with age in rats: role of glucocorticoids. *Endocrinology*., **117**, 662-6.

Salak-Johnson, J.L. and McGlone, J.J. (2007). Making sense of apparently conflicting data: stress and immunity in swine and cattle. *J Anim Sci*., **85**, E81-8.

Sareh H., Tulapurkar, M.E., Shah, N.G., Singh, I.S. and Hasday, J.D. (2011). Response of mice to continuous 5-day passive hyperthermia resembles human heat acclimation. *Cell Stress Chaperones.*, **16**, 297-307.

Sei, Y., Skolnick, P. and Arora, P.K. (1992). Strain variation in immune response and behavior following the death of cage cohorts. *Int J Neurosci*., **65**, 247-58.

Shavit, Y., Depaulis, A., Martin, F.C., Terman, G.W., Pechnick, R.N., Zane, C.J, Gale, R.P. and Liebeskind, J.C. (1986). Involvement of brain opiate receptors in the immune-suppressive effect of morphine. *Proc Natl Acad Sci U S A*., **83,** 7114-7.

Solov'ev, A.S. (1992). The effect of a high external temperature on cellular immunity. *Biull Eksp Biol Med*., **114**, 382-3.

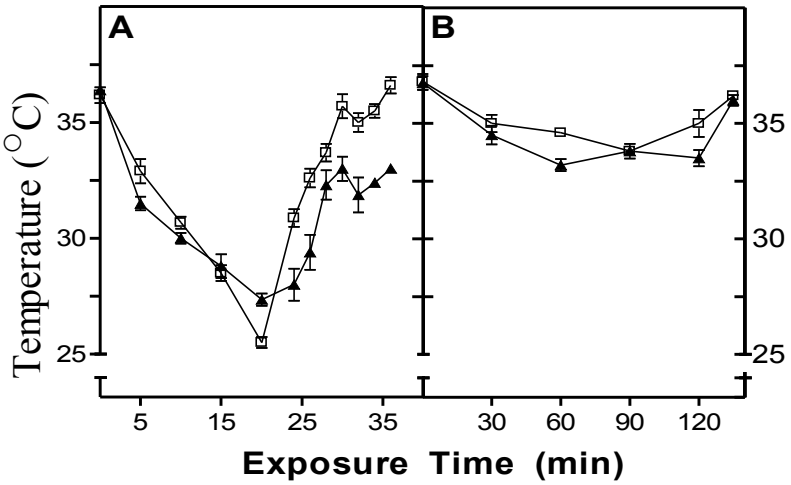
Steplewski, Z. and Vogel, W.H. (1986). Total leukocytes, T cell subpopulation and natural killer (NK) cell activity in rats exposed to restraint stress. *Life Sci*., **38**, 2419-27.

Tecoma, E.S. and Huey, L.Y. (1985). Psychic distress and the immune response. *Life Sci*., **36**, 1799-812.

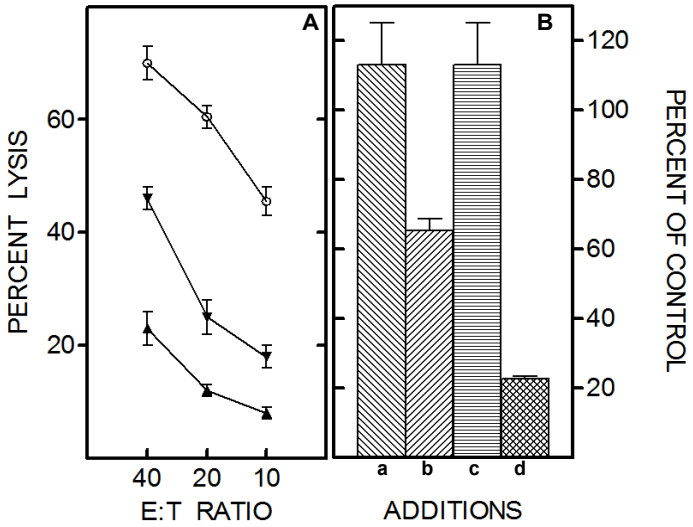
Wetsel, W.C. (2011). Hyperthermic effects on behavior. *Int J Hyperthermia*., **27**, 353-73.

Yoshioka, A., Miyachi, Y., Toda, K., Imamura, S., Hiraoka, M. and Abe, M. (1990). Effects of local hyperthermia on natural killer activity in mice. *Int J Hyperthermia*., **6**, 261-7.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Body temperature in cold exposed and immobilized mice. C57BL/6 (▲) or BALB/c (□) mice were subjected to cold (Panel A) and immobilization stress (Panel B) for 20 min and 120 min respectively. Mice were individually subjected to cold in a plastic box (23 x 12 x 11 cm) that was placed at -20°C for 20 min. Mice were individually subjected to immobilization for 2 hr in a capped 30 ml plastic tissue culture tube (2.4 x 9.3 cm) that was perforated to allow free breathing. Rectal temperatures were recorded in both strains of mice at indicated time intervals and represented as mean rectal temperature ± SD for 3 mice per group.



Supplementary Figure 2. Effect of serum obtained from heat exposed and unexposed mice on CTL generation and CTLL-2 proliferation. Panel A. Splenocytes obtained from primed normal C57BL/6 mice were restimulated in vitro with irradiated BALB/c splenocytes in the presence of medium alone (○), medium supplemented with 6% serum from heat exposed C57BL/6 mice (▲) and medium supplemented with 6% serum from heat exposed BALB/c mice (▼). Effector cells obtained after 5 day culture were assayed on P815 targets in a 5 hr chromium release assay. Data is represented as percent lysis obtained at different E:T ratios ± SD. Panel B: CTLL-2 cells were cultured in medium containing 0.2 U/ml IL-2, in the absence (control) or presence of 9% serum equivalent retentate (histograms b and c) or filtrate (histograms a and c) obtained from the serum of normal unexposed (histogram b) or heat exposed (histogram c) C57BL/6 mice. All cells were pulsed with 3[H] thymidine and harvested as described in Materials and Methods section. All assays were performed in triplicates and results are expressed as percent of control response ± SD of triplicates.