Attenuated Thermoregulatory, Metabolic and Liver Acute Phase Protein Response to Heat Stroke in TNF Receptor Knockout Mice

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ABSTRACT

Tumor necrosis factor (TNF) is considered an adverse mediator of heat stroke (HS) based on clinical studies showing high serum levels. However, soluble TNF receptors (sTNFR; TNF antagonists) were higher in survivors than nonsurvivors and TNFR KO mice showed a trend towards increased mortality suggesting TNF has protective actions for recovery. We delineated TNF actions in HS by comparing thermoregulatory, metabolic and inflammatory responses between B6129F2 (WT) and TNFR KO mice. Prior to heat exposure, TNFR KO mice showed ~0.4°C lower core temperature (Tc; radiotelemetry), ~10% lower metabolic rate (Mr; indirect calorimetry) and reduced plasma IL-1α and sIL-1RI than WT mice. KO mice selected warmer temperatures than WT mice in a gradient, but remained hypothermic. In the calorimeter, both genotypes showed a similar heating rate, but TNFR KO maintained lower Tc and Mr than WT mice for a given heat exposure duration and required ~30 min longer to reach maximum Tc (42.4°C). Plasma IL-6 increased at ~3h of recovery in both genotypes, but KO mice showed a more robust sIL-6R response. Higher sIL-6R in the KO mice was associated with delayed liver p-STAT3 protein expression and attenuated serum amyloid A3 (SAA3) gene expression suggesting the acute phase response (APR) was attenuated in these mice. Our data suggest that the absence of TNF signaling induced a regulated hypothermic state in the KO mice, TNF-IL-1 interactions may modulate Tc and Mr during homeostatic conditions, and TNF modulates the APR during HS recovery through interactions with the liver IL-6-STAT3 pathway of SAA3 regulation.

Key words: TNF; IL-6; heat stress; hyperthermia; acute phase response
INTRODUCTION

Heat stroke is characterized by severe hyperthermia and development of a systemic inflammatory response syndrome (SIRS) that often progresses to multi-organ damage and death (27). Tumor necrosis factor (TNF) has been implicated as an adverse mediator of the heat-induced SIRS based almost exclusively on clinical studies showing high plasma levels in heat stroke patients at the time of collapse or shortly after cooling (5, 19). However, several studies have failed to show a correlation between high circulating TNFα levels and hyperthermia, endotoxemia or other circulating inflammatory cytokines (e.g., interleukin [IL]-1 or IL-6) that have been implicated in the SIRS (5, 6, 19). Results from a small clinical cohort study (N=3) support the hypothesis that TNF has detrimental effects during heat stroke recovery since survivors showed higher circulating levels of the soluble TNF type II receptor (sTNFRII, a naturally occurring antagonist of TNF; (24)) than non-survivors (10). However, the small sample size of that clinical study, inconsistencies in the ability to detect circulating TNFα and poor correlation with Tc and other SIRS responses have limited our understanding of the actions of this cytokine in the heat stroke syndrome.

TNF inhibition/neutralization studies in experimental animal models have also provided conflicting interpretation of TNF actions in heat stroke. Smith et al. (28) examined the effect of a peripheral injection of infliximab (a TNFα monoclonal antibody) on core temperature (Tc), gut damage and survival of rats following ~70 min heat exposure. Plasma TNFα was undetectable at the end of heat exposure in both vehicle- and infliximab-treated rats and the antibody had no effect on maximum Tc (Tc,Max=43.0°C) or other heat stroke responses. We showed in mice that
plasma TNFα was either unchanged or decreased at $T_{c,\text{Max}}$ and later time points of heat stroke recovery (7, 15, 30), yet robust increases in sTNFRI and II were observed throughout recovery (2). Plasma sTNFRs have been shown to protect against TNFα-induced cytokine production and cytotoxicity (29), which is congruent with the purported protective actions of the soluble receptors in heat stroke patients (10). Based on preliminary data from a mouse heat stroke model used in our laboratory, we propose the alternative hypothesis that TNF has both pro- and anti-inflammatory actions that are time- and context-dependent (i.e., dependent on interactions with other cytokines). This is suggested by TNF p55 (type I)/p75 (type II) receptor knockout (TNFR KO) mice that displayed longer heating times to $T_{c,\text{Max}}$ and more rapid cooling (both protective $T_c$ responses), but showed a trend towards increased mortality compared to wild-type mice during the second day of recovery (14).

The goal of the current study was to delineate the actions of TNF in the heat stroke syndrome by comparing thermoregulatory, metabolic and inflammatory responses between B6129F2 and TNFR KO mice. We first examined thermoregulatory and metabolic responses of mice under normal housing conditions to determine if the effects of TNF neutralization on $T_c$ responses was specific to heat stress or represented a general effect on all thermoregulatory processes. Metabolic rate ($M_r$) was measured due to reliance of mice on this autonomic mechanism for $T_c$ regulation (23). We then tested the hypothesis that the slower heating rates previously observed in TNFR KO mice were a consequence of a lack of TNF stimulation on $M_r$ during heat exposure. $M_r$ and $T_c$ responses of B6129F2 and TNFR KO mice were compared during heat exposure to the same $T_{c,\text{Max}}$ in an indirect calorimeter. To determine the effect of TNF neutralization on the SIRS, we examined circulating cytokine / soluble cytokine receptor responses, liver cytokine signaling and acute phase protein (APP) gene expression during the
early stages of heat stroke recovery. We focused on the liver due to the complex network of cytokine interactions that are thought to mediate the acute phase response (APR) to systemic inflammation during heat stroke recovery (11).

MATERIALS AND METHODS

Animals. Specific pathogen-free male B6129F2 (B6129SF2/J; Jackson Laboratories, Bar Harbor, ME) and TNFR KO (B6;129S-Tnfrsf1a<sup>tm1lmx</sup> Tnfrsf1b<sup>tm1lmx</sup>/J; Jackson Laboratories, Bar Harbor, ME) mice weighing 26.9 ± 1.0g were used (N=143 mice). Mice were individually housed in Nalgene polycarbonate cages (11.5 in. x 7.5 in. x 5 in.) fitted with Hepa-filter cage tops and wood chip bedding (Pro-Chip, PWI, Canada). Rodent laboratory chow (Harlan Teklad, LM-485, Madison, WI) and water were provided ad libitum under standard laboratory conditions (25 ± 2°C; 12:12h light-dark cycle, lights on at 0600h). Environmental enrichment was provided in each cage as a Nalgene Mouse House® (Nalgene Nunc Int., Rochester, NY) with a maple wood product (Product #W0002, Bio-Serv, Frenchtown, NJ) and stainless steel ring attached to the wire lid to encourage foraging and climbing behaviors, respectively. Fresh cages, food and water were provided on a weekly schedule. In conducting research using animals, we adhered to the Guide for the Care and Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. All procedures received Institutional Animal Care and Use Committee approval prior to experimentation.

Radiotelemetry Transmitter Implantation. Two types of radiotelemetry transmitters were used in this study. For circadian and behavioral thermoregulatory experiments, mice were intraperitoneally implanted with a free-floating battery-operated transmitter (3.5g, volume 1.75 cc; model TA10TA-F20, Data Sciences International, St. Paul, MN). For all other experiments,
mice were implanted with a battery-free radiotelemetry transmitter (1.1g, volume 0.52cc; model G2 Emitter, Mini Mitter Co., Inc., Bend, OR). Both transmitters allowed remote measurement of $T_c (\pm 0.1^\circ C)$ and activity (counts) in conscious, unrestrained mice. Mice were anesthetized with isoflurane and the abdominal fur was shaved and scrubbed with a 10% Povidone-iodine solution (Betadine® Solution, Purdue Frederick Co., Stamford, CT) followed by 70% isopropyl alcohol. Each radiotelemetry device was disinfected by pre-soaking for 1h in cold sterilant (Actril®, Minntech Corporation, Minneapolis, MN) followed by three rinses in sterile saline (0.9%). A ~1 cm incision was made through the skin and abdominal muscle layer using aseptic technique and the transmitter was placed among the abdominal organs with the G2 transmitter model sutured to the peritoneal muscle layer (as recommended by the manufacturer) using non-absorbable suture (6-0 Prolene, P-1 Reverse Cutting, Ethicon, Somerville, NJ). The peritoneal muscle and skin layers were closed with absorbable suture (5-0 Coated Vicryl, RB-1 Taper, Ethicon, Somerville, NJ) in interrupted and continuous subcuticular patterns, respectively. Immediately following surgery, each mouse was placed into a clean cage with ad libitum food and water and returned to the animal room for undisturbed recovery. $T_c$ and activity were continuously monitored at 1-min intervals using the Vitalview® (G2 emitter; Mini Mitter Co., Inc., Bend, OR) or Dataquest Data Acquisition system (TA10TA-F20 model, Data Sciences International, St. Paul, MN). Each transmitter emitted a unique frequency (Hz) that was proportional to $T_c$ and detected by an antenna under the animal's cage; frequency values were transferred to a peripheral PC and converted to $T_c$ using predetermined calibration values. Activity was detected as changes in signal strength and represented a general measure that did not distinguish between motor movements and postural changes.
**Surgical Analgesia.** Analgesia was provided as a 190 mg Dustless Precision Pellet® (Product #F6698, F6699, F6700, and F6701; Bio-Serv, Frenchtown, NJ) that contained 25 μg of indomethacin (Sigma #I-8280, St. Louis, MO). Pellets were placed onto the cage floor for voluntary consumption ~1h prior to surgery and at 0800h on days 1, 2 and 3 of recovery. Consumption of the pellet was visually confirmed. Surgical recovery was achieved at ~1 week, as defined by a return to pre-surgical body weight (minus transmitter weight), re-establishment of normal food and water consumption and stable circadian $T_c$ and activity, as previously described (18).

**Heat Stress Protocol.** The heat stress protocol has been described in detail elsewhere (16). Briefly, ~24h prior to heat exposure, mice in their home cages with food, water, and bedding were placed into a floor-standing incubator (Therma Forma, Model 3950, Marietta, OH) at the normal housing temperature of $25 \pm 2^\circ$C to acclimate to the fan noises. Cage filter tops were removed to facilitate air circulation. The following day between ~0800 and 1000h, mice with baseline $T_c<36.0^\circ$C were weighed, food, water and enrichment products were removed from the cage and the incubator ambient temperature ($T_a$) was increased to $39.5 \pm 0.2^\circ$C (required ~1h to reach this $T_a$). Mice remained in the heat stress environment until $T_{c,\text{Max}}$ of 42.4°C was reached. At $T_{c,\text{Max}}$, mice were removed from the heat, body weight was recorded and food and water placed back into the cage for undisturbed recovery at $25 \pm 2^\circ$C. Non-heated control mice were exposed to the same conditions at $T_a$ of $25 \pm 2^\circ$C with the timing of experimental procedures matched to that of a heat-exposed mouse.

The experimental protocol for this study is depicted in Figure 1 with details provided below.

**Experiment 1: Circadian and Behavioral Thermoregulatory Profiles of B6129F2 and TNFR KO Mice.** Following ≥1 week recovery from transmitter implantation, circadian $T_c$ and activity
profiles of B6129F2 and TNFR KO mice (N=10 mice/genotype) were examined under normal housing conditions (0600 to 0559h; Figure 1A). Mice remained in their original cage throughout experimentation with Tc and activity recorded at 1-min intervals. Following assessment of circadian Tc and activity profiles, mice were placed into a temperature gradient to compare behavioral thermoregulatory responses between strains. Each mouse was placed into the temperature gradient ≥16-h prior to experimentation to minimize confounding stress effects (e.g., hyperthermia, increased activity) during initial exposure. Behavioral thermoregulatory responses were analyzed across the subsequent 24-h period (0600-0559h; Figure 1A). The thermal gradient has been described in detail elsewhere (9). Briefly, the gradient runway was constructed of a series of copper bars cooled or heated by water circulating through copper tubing that encompassed opposite ends of the runway. Runway temperature ranged from ~18-40°C along the length of the gradient. Perforated stainless steel walls confined the mouse to the runway while maintaining adequate air circulation and entrainment to the L:D cycle. The position of the mouse was determined by photocell emitters / detectors along the runway and reported as 1-min selected temperature (Ts) values from copper-constantan thermocouples inserted into the runway at each location along the gradient length. Food and water were provided ad libitum at the two ends and middle of the gradient to minimize influence of food and water availability on Ts. Three equally spaced radiotelemetry wand receivers (Model RLA-3000, Data Sciences, St. Paul, MN) along the top of the gradient monitored Tc at 1-min intervals.

Experiment 2: Tc and Mr of B6129F2 and TNFR KO Mice during Heat Exposure in an Indirect Calorimeter. These studies were conducted in a separate group of mice (N=8/genotype) than those used for circadian and behavioral thermoregulatory analyses. Following recovery from transmitter surgery (≥1 week), mice were placed into the calorimeter chamber at Ta of 25°C.
with food and water to acclimate for $\geq 16\text{h}$ to this novel environment (Figure 1B). Starting at

$\sim 0800$-$1000\text{h}$ the next day, $T_c$ and $M_r$ of non-heated control mice were examined in the indirect

calorimeter at $T_a$ of $25\pm 2^\circ\text{C}$ for $\sim 4\text{ hours}$ (represents the average heat exposure time for mice to

reach $T_{c,\text{Max}}$ of $42.4^\circ\text{C}$; (14, 16)). Following control experimentation, mice were allowed to

recover for $\geq 1\text{ week}$ at the $T_a$ of $25\pm 2^\circ\text{C}$ and were then re-acclimated to the calorimeter chamber

for $\geq 16\text{ hours}$ and then tested in the heat stroke condition, using the heat stress protocol

described above (Figure 1B). Mouse $M_r$ and $T_c$ responses were measured in a sealed clear

plexiglass cage (8 in. x 5 in. x 4 in.) maintained in a temperature-controlled chamber ($39.5 \pm

0.5^\circ\text{C}$; Powers Scientific, Inc., Pipersville, PA). Oxygen consumption ($V_O_2; \text{ml/h/g}$) and carbon

dioxide production ($V_C_O_2; \text{ml/h/g}$) were determined in a Comprehensive Lab Animal

Monitoring System interfaced with Oxymax software (CLAMS, Columbus Instruments,

Columbus, OH). $M_r$ ($\text{kcal/hr}$) was automatically calculated by the Oxymax software and

converted to $\text{W/kg/h}$ ($[\text{kcal/hr}*1.161]/\text{kg BW}$) for presentation. Compressed air of known $O_2$ and

$CO_2$ concentration was flushed through the calorimeter chamber at a rate of $0.5\text{ L/min}$ (as

recommended by the manufacturer) and the expired chamber air was dessicated by drawing it

through a canister of Drierite. The dried air was sampled at 1-min intervals by high-speed gas

sensing $O_2$ and $CO_2$ analyzers ($\pm 0.002\%$), which were calibrated prior to each experiment by

flushing a calibration gas of known concentration ($20.5\% O_2, 0.5\% CO_2$, balance $N_2$) through the

chamber to ensure measurement accuracy. Bedding was not provided in the calorimeter chamber

due to the potential trapping of air that would interfere with gas measurements. $T_c$ values were

recorded at 1-min intervals by a radiotelemetry antenna system integrated into the CLAMS

system.
Experiment 3: Plasma Cytokine, Soluble Cytokine Receptor and Liver APP Expression of B6129F2 and TNFR KO Mice during Heat Stroke Recovery. Blood and liver were collected from separate groups of mice than those described above. Following recovery from transmitter implantation (≥1 week), non-heated control and heat stroke mice were placed into the heating chamber (i.e., floor-standing incubator) to acclimate for >16-h to this environment at Ta of 25°C (Figure 1C). Starting between ~0800-1000h the next day, mice were randomly assigned to the following groups and sacrificed at each time point for blood and liver collection with non-heated controls matched to a heat stroke mouse at each time point (Figure 1C): (I) baseline (Tc<36.0°C; immediately prior to heat stress); B6129F2, n=10, TNFR KO, n=8; (II) Tc,Max; B6129F2 non-heated control, n=7, TNFR KO non-heated control, n=6, B6129F2 heat, n=7, TNFR KO heat, n=6; (III) return-to-baseline (RTB; first Tc value <36.0°C during cooling); B6129F2 non-heated control, n=8, TNFR KO non-heated control, n=8, B6129F2 heat, n=8, TNFR KO heat, n=9; or (IV) hypothermia depth (HD; lowest 1-min Tc value during recovery with 0.01°C/min cooling rate); B6129F2 non-heated control, n=6, TNFR KO non-heated control, n=8, B6129F2 heat, n=8, TNFR KO heat, n=8. Tc was monitored at 1-min intervals throughout experimentation.

Blood and Tissue Harvesting. Mice were deeply anesthetized with isoflurane and exsanguinated following thoracotomy and intracardiac puncture (1 ml syringe, 23 ga. needle). Blood was transferred to 1.5 ml EDTA microcentrifuge tubes and immediately placed onto ice until plasma was separated by centrifugation (4°C; 5 min, ~2800 rcf). Following exsanguination mice were infused with cold heparinized (10 U/ml) sterile saline and the liver rapidly excised, frozen in liquid nitrogen, and stored at -80°C until analysis.

Plasma Protein Measurements. Mouse custom plex kits were used to analyze duplicate plasma samples for IL-1α, IL-1β, IL-6, IL-10, TNFα (Bio-Rad Laboratories, Ind., Hercules,
Mechanisms of TNF actions in Heat Stroke

CA), sgp130, sIL-1RI, sIL-1RII, sIL-6R, sTNFRI, sTNFRII (Millipore, Corporation, Billerica, MA) and liver phosphorylated signal transducer and activator of transcription 3 (STAT3; Try305; Bio-Rad Laboratories, Ind., Hercules, CA) using the FlowMetrix™ System (Luminex, Austin, TX). Due to limited plasma volume, soluble cytokine receptor analysis could only be performed on a subset of mouse samples (n=5 to 8 mice/group) for which plasma was available after cytokine analysis. For liver p-STAT3 measurement, ~50 mg frozen liver samples were homogenized in 500 µl cell lysis buffer containing protease inhibitor cocktail (171-304012; Bio-Rad, Hercules, CA) and 2 mM phenylmethylsulfonyl fluoride (P-7626) in dimethyl sulphoxide (D-2650; both from Sigma-Aldrich, St. Louis, MO). Homogenates were sonicated on ice and centrifuged at 4500 g for 5 min at 4°C. Supernatant total protein concentration was determined using the BCA Protein Assay (23225; Thermo Scientific, Rockford, IL) and samples were diluted with cell lysis buffer to a final protein concentration of 500 µg/ml. Cytokines and soluble cytokine levels are reported as pg/ml and STAT3 levels as mean fluorescent intensity (MFI).

Western Blot Measurements. Liver HSP70 protein levels were measured in equal amounts of protein (60 µg / sample) from homogenized liver samples that were separated by SDS/PAGE and blotted to polyvinylidene fluoride (PVDF) membranes. After blocking with Odyssey blocking buffer (927-40000; LI-COR, Lincoln, NE), membranes were incubated with rabbit polyclonal anti-HSP70 (SPA-812; Enzo Life Sciences, Farmingdale, NY) and goat polyclonal anti-GAPDH (for normalization of protein load; SAB2500450-100UG; Sigma-Aldrich, St. Louis, MO) primary antibodies. Subsequently, membranes were incubated with LI-COR infrared labeled secondary antibodies, donkey anti-rabbit IRDye 800CW and anti-goat IRDye 680LT to bind the primary antibodies. The bound complex was detected using the LI-COR Odyssey Infrared
Imaging System and images were analyzed using the Odyssey Application Software (ver. 3.0) to obtain normalized integrated intensities.

RNA extraction, reverse transcription and real-time PCR. Gene expression levels of murine serum amyloid A (SAA1), SAA3, SAA4, haptoglobin (Hp), fibrinogen A (FgA), FgB, FgG, β-glucuronidase (Gusb), phosphoglycerate kinase-1 (PGK-1) and 18s RNA (Applied Biosystems, Foster City, CA) were determined from RNA that was isolated from ~30 mg frozen B6129F2 and TNFR KO liver samples collected at hypothermia depth only. RNA purity for all samples was ≥1.8 and ≥1.95 for the 260/230 and 260/280 nm ratios, respectively.

RNA samples were reverse-transcribed into cDNA (37°C for 2h) using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). TaqMan Expression Assays containing 0.9 μM of each primer and 0.25 μM of a TaqMan MGB probe, comprising FAM reporter dye at the 5’-end and a non-fluorescent quencher at the 3’-end, were combined with 50 ng cDNA, Fast Advanced Master Mix and water in a final reaction volume of 20 μl. RT-PCR reactions were performed in duplicate under the following conditions: hold at 95°C for 20s, 40 cycles of 95°C for 1s and 60°C for 20s (StepOne Plus Real-Time PCR System; Applied Biosystems) with >90% efficiency for all genes. To ensure specificity of primer combinations, PCR products from randomly chosen reactions were separated on a 1% agarose gel revealing the presence of a single band for each PCR product. Details of gene expression analysis are described elsewhere (11).

Calculations. Body weight was determined on a top-loading balance (±0.1g) and corrected for transmitter weight. Percent dehydration induced by heat exposure was calculated as [(pre heat stress BW – Tc,Max BW) / pre heat stress BW] x 100, which represents an estimation that did not account for BW loss from feces or urine. Thermal load (°C·Min) was calculated as Σ of the
Mechanisms of TNF actions in Heat Stroke

\[ \text{time intervals (min) x (°C above } \text{T}_c=39.5°C \text{ at the start of the interval + °C above } \text{T}_c=39.5°C \text{ at the end of the interval)/2.} \]

\[ \text{Time to } \text{T}_{c,\text{Max}} \text{ (min) represents the time from the start of heat stress to } \text{T}_{c,\text{Max}} \text{ of 42.4°C; cooling time to baseline (min) represents the time from } \text{T}_{c,\text{Max}} \text{ to the first } \text{T}_c \text{ value <36.0°C during cooling; time to HD (min) represents the time from } \text{T}_{c,\text{Max}} \text{ to the lowest 1-min } \text{T}_c \text{ value during recovery; HD (°C) represents the lowest } \text{T}_c \text{ value observed during cooling, which was associated with 0.01°C/min cooling rate, as previously described for our mouse heat stroke model (15).} \]

**Genotyping.** At the end of blood and organ harvesting, ~1 cm of tail was obtained from each mouse for genotyping. Tails were digested using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) per manufacturer instructions. PCR was performed using murine primer sets designed against TNFRI (p55) and II (p75) (Operon, Huntsville, AL). Resultant PCR products were run on 1% agarose gels to compare B6129F2 and TNFR KO banding patterns and confirm the wild-type and mutant genotypes.

**Data Analysis.** \( \text{T}_c, \) activity, \( \text{T}_s, \) \( \text{Mr}, \) and liver HSP70 protein data are means ± SE. Plasma cytokine, soluble cytokine receptor and APP gene expression values are presented as mean fold-change without SE. p-STAT3 data are presented as MFI ± SE. \( \text{T}_c \) and activity are presented as 1-h averages (circadian and behavioral thermoregulatory responses) or 10 min averages (indirect calorimeter experiment only). \( \text{Mr} \) was determined at 2- and 5-min intervals and averaged into 10-min bins for presentation. Non-heated control mice showed virtually identical plasma and liver gene / protein profiles across all time points; these data were statistically analyzed between heat stroke mice and their time-matched controls at each time point, but for ease of presentation, non-heated control values are graphically presented for baseline only. Time to \( \text{T}_{c,\text{Max}} \), thermal load, cooling time to baseline, time to HD, and SAA3 fold-change were analyzed using One-
Mechanisms of TNF actions in Heat Stroke

Way Analysis of Variance (ANOVA). All other measurements were analyzed using a Two-Way ANOVA. The Holm-Sidak method was used as the post-hoc test for multiple comparisons with significance set at P<0.05. All statistical analyses were performed using the SigmaPlot 12.0 software (SysStat Software Inc., San Jose, CA).

RESULTS

Experiment 1: Circadian and Behavioral Thermoregulatory Profiles of B6129F2 and TNFR KO Mice.

During housing at Ta of 25°C, B6129F2 and TNFR KO mice displayed circadian Tc and activity rhythms with low daytime (lights-on, inactive period) and high nighttime (lights-off, active period) values (Figure 2A and B). TNFR KO mice maintained ~0.4°C lower daytime Tc than B6129F2 mice from 0700 to 1600h (35.9 ± 0.1°C vs. 36.2 ± 0.1°C, respectively; ANOVA, P<0.001; Figure 2A) whereas activity levels were similar between genotypes during this time (5.0 ± 1.0 vs. 5.3 ± 1.1, respectively; Figure 2B). Nighttime Tc and activity were similar between genotypes with a peak from 1800 to 2200h followed by a gradual return to baseline levels (Figure 2A and B).

The circadian rhythm displayed by B6129F2 and TNFR KO mice in the temperature gradient consisted of warm daytime Ts when Tc was low and cooler nighttime Ts when Tc was elevated (Figure 3A and B). TNFR KO mice maintained ~0.4°C lower daytime Tc than B6129F2 mice from 0600 to 1500h (35.9 ± 0.0°C vs. 36.3 ± 0.2°C, respectively; ANOVA, P<0.001) despite residing at ~2°C warmer Ts during this time period (0600-1300h: 32.0 ± 0.2°C vs. 30.4 ± 0.6°C, respectively; ANOVA, P<0.001; Figure 3A and B). Ts of TNFR KO mice was higher than B6129F2 mice from 2100-0100h, but Tc did not differ between genotypes during this time (Figure 3A and B).
Experiment 2: Tc and Mr of B6129F2 and TNFR KO Mice during Heat Exposure in an Indirect Calorimeter. Figure 4 shows Tc and Mr of B6129F2 and TNFR KO mice during non-heated control (A and B) and heat stroke experimentation (C and D) in the calorimeter. B6129F2 and TNFR KO responses were graphed through ~210 and ~270 min of experimentation only, which represents the time that the first mouse of each genotype reached Tc,Max, respectively. Prior to experimentation, TNFR KO mice maintained ~0.8°C lower Tc and ~10% lower Mr than B6129F2 mice (B6129F2: 36.5±0.3°C; TNFR KO: 35.7±0.2°C; Figure 4). Weighing and handling at time 0 induced ~2°C stress-hyperthermia that was associated with a robust increase in Mr from ~0-60 min that did not differ between genotypes during control (Figure 4A and B) or heat stroke experimentation (Figure 4C and D). During control experimentation, Tc and Mr of B6129F2 mice was higher than TNFR KO mice with the exception of ~130-170 min when TNFR KO mice showed a transient increase presumably due to human disturbance in the animal room (Figure 4A and B). From ~70-210 min of heat exposure, TNFR KO mice maintained ~0.8°C lower Tc and ~15% lower Mr than B6129F2 mice (ANOVA, P<0.001; Figure 4C and D), which resulted in TNFR KO mice requiring ~30 min longer to attain Tc,Max. As a result of this longer heat exposure time, TNFR KO mice experienced a higher level of dehydration compared to B6129F2 mice (314 ± 10 min, 11 ± 0.6%, vs. 279 ± 14 min, 8 ± 1%, respectively; P<0.05).

Experiment 3: Plasma Cytokine, Soluble Cytokine Receptor and Liver APP Expression of B6129F2 and TNFR KO Mice during Heat Stroke Recovery. Table 1 describes the thermoregulatory responses of B6129F2 and TNFR KO mice that were sacrificed at baseline, Tc,Max, RTB or HD. Non-heated control mice of both genotypes experienced ~2.5% dehydration, presumably due to the absence of water during experimentation (Table 1). Both genotypes showed heat exposure responses similar to those observed in the calorimeter with TNFR KO
mice requiring ~30 min longer to reach $T_{c,\text{Max}}$ (ANOVA, $P=0.007$; Table 1). The increased heat exposure time of TNFR KO mice was associated with significantly greater dehydration than B6129F$_2$ mice (11.8 ± 0.4 vs. 10.0 ± 0.5%, respectively; ANOVA, $P<0.001$; Table 1) whereas thermal load was similar between genotypes (ANOVA, $P=0.252$; Table 1). During recovery, TNFR KO mice cooled more rapidly, but HD was similar between genotypes at ~31°C (ANOVA, $P=0.471$; Table 1). Figure 5 shows the sampling time points for blood and liver collection relative to the $T_c$ responses displayed by each genotype during heat exposure and recovery.

Prior to heat exposure (baseline, *time 0*), TNFR KO mice showed significantly lower plasma IL-1$\alpha$ levels than B6129F$_2$ mice (Figure 6A) whereas baseline levels of plasma IL-1$\beta$ (Figure 6B), IL-6 (Figure 6C) and IL-10 (Figure 6D) were similar between genotypes. On the other hand, the plasma baseline level of TNF$\alpha$ was significantly higher in TNFR KO than B6129F$_2$ mice (2304.5 ± 350.7 vs. 409.3 ± 8.3 pg/ml; ANOVA, $P<0.001$; Figure 6E). Plasma IL-1$\alpha$ and IL-1$\beta$ did not change during heat stroke recovery in either genotype (Figure 6A and B). Plasma IL-6 was not increased in either genotype until HD with ~2-3-fold increase above non-heated controls in both genotypes (ANOVA, $P<0.05$; Figure 6C). The only increase in plasma IL-10 was observed at HD in TNFR KO mice and this represented a significant difference between genotypes at this time point (156.5 ± 56.7 pg/ml vs. 500.5 ± 129.2; ANOVA, $P=0.016$; Figure 6D). TNFR KO mice maintained significantly higher plasma TNF$\alpha$ levels compared to B619F$_2$ mice throughout recovery, but heat stroke had no effect on this cytokine (ANOVA, $P<0.001$; Figure 6E).
Plasma sgp130 (Figure 7A) and sIL-1RII (Figure 7C) were similar between genotypes at all sampling time points. TNFR KO mice showed significantly lower sIL-1RI levels at baseline compared to B6129F2 mice (2950 ± 151 vs. 5140 ± 862 pg/ml; ANOVA, P=0.031), but this soluble cytokine receptor did not change in either genotype during heat stroke recovery (Figure 7B). Baseline levels of the sIL-6R were similar between genotypes, but TNFR KO mice showed an increase in sIL-6R above controls from RTB to HD that was not observed in B6129F2 mice (%ANOVA, P<0.001; Figure 7D). B6129F2 mice showed ~4-fold higher sTNFRII than I levels at baseline (8777 ± 769 pg/ml vs. 2190 ± 59, respectively; Figure 7E and F). B6129F2 mice showed increased plasma sTNFRI and II levels compared to non-heated controls from Tc,Max to HD (%ANOVA, P<0.001), whereas the sTNFRs were undetectable at all time points in the TNFR KO mice (Figure 7E and F). Genotype analysis confirmed the absence of TNFRI and II in the KO mice (data not shown).

Liver HSP70 protein levels were similar between genotypes at baseline (Figure 8A and B). Both genotypes showed a significant increase in liver HSP70 at HD, which was virtually identical between groups, but represented a significant increase compared to the earlier time points of recovery (%ANOVA, P<0.05; Figure 8).

B6129F2 mice showed increased liver p-STAT3 levels from RTB through HD whereas this response was delayed until HD in TNFR KO mice (%ANOVA, P<0.05; Figure 9A). The only significant difference between groups was observed at RTB with p-STAT3 higher in B6129F2 than TNFR KO mice (34.4 ± 6.1 vs. 18.5 ± 3.6 MFI, respectively; %ANOVA, P<0.05; Figure 9A). SAA1, SAA4, Hp, FgA, FgB and FgG gene expression was not increased in B6129F2 or TNFR KO mice at HD (only time point measured; data not shown). Both genotypes showed a significant increase in liver SAA3 gene expression above non-heated controls at HD, which was
Mechanisms of TNF actions in Heat Stroke

more pronounced in B6129F2 compared to TNFR KO mice (3.8- vs. 2.7-fold, respectively; ANOVA, P=0.009; Figure 9B).

DISCUSSION

The goal of this study was to delineate TNF actions in the heat stroke syndrome by comparing thermoregulatory, metabolic and inflammatory responses between B6129F2 and TNFR KO mice. We first compared Tc, Mr and inflammatory responses between strains during non-heat stress conditions to examine the role of TNF in circadian Tc control. TNFR KO mice showed ~0.4°C lower daytime Tc than B6129F2 mice during housing at T_a of 25°C and this Tc difference was maintained in the gradient despite the selection of warmer temperatures by the KO mice. The lower daytime Tc of the TNFR KO mice was not a consequence of reduced motor activity since both strains showed similar circadian activity profiles. However, baseline plasma IL-1α, sIL-1RI and Mr were lower in the KO compared to wild-type mice suggesting TNF-IL-1 interactions may modulate Tc and/or Mr under resting conditions. In the calorimeter, the Tc and Mr difference between genotypes was maintained and resulted in a longer duration of heat exposure before TNFR KO mice reached the Tc,Max of 42.4°C. During heat stroke recovery, TNFR KO mice showed higher plasma sIL-6R with attenuated liver p-STAT3 and SAA3 responses compared to B6129F2 mice suggesting IL-6 regulation of the APR may be compromised in these animals.

During housing at T_a of 25°C, TNFR KO mice maintained stable, albeit lower daytime Tc than B6129F2 mice while activity was virtually identical between genotypes. Because mice and other small rodents rely primarily on metabolic adjustments for Tc control (23), these findings suggest that neutralization of TNF signaling induced a forced reduction in Tc due to a deficit in
metabolic thermogenesis. To determine if the lower $T_c$ of TNFR KO mice was due to a metabolic deficiency or a decrease in the temperature set point (i.e., an effect on CNS mechanisms of $T_c$ control), we examined behavioral responses in a gradient that ranged in temperature from ~18-40°C. If the lower $T_c$ of the KO mice was a forced response due to a defect in metabolic control, we expected TNFR KO mice to select gradient temperatures of 39.5°C or higher to normalize $T_c$. This hypothesis was based on the observation that $T_a$ of 39.5°C was sufficient to reverse the hypothermia of TNFR KO mice using our heat stress protocol (current study and (14)). As shown in the gradient experiment, the set point for $T_c$ control appeared to be reduced in the TNFR KO mice since they selected runway temperatures of ~32°C, which were higher than those selected by B6129F2 mice, but not warm enough to reverse the hypothermia. Rather, TNFR KO mice maintained $T_c$ of ~35.9°C in the gradient, which was virtually identical to that observed under normal housing conditions. The maintenance of similar daytime $T_c$ under conditions in which a deficit in autonomic thermoeffectors could have been compensated for by thermoregulatory behavior suggests that these mechanisms of $T_c$ control were operating similarly to induce a regulated state of hypothermia in the KO mice. Additionally, TNFR KO mice do not appear to have a metabolic deficiency since they were shown previously to develop a similar magnitude of fever (~1°C) as B6129F2 mice the day following heat exposure (14, 17). This delayed fever is a regulated $T_c$ response (i.e., due to an elevation in the temperature set point) that is associated with ~20% increase in $M_t$ (17) The ability of TNFR KO mice to activate $T_c$ and $M_t$ to invoke a similar magnitude of fever as B6129F2 mice indicates these mice are not metabolically deficient, but simply residing at a lower baseline $T_c$ than their wild-type counterparts.
The hypothermic state of the TNFR KO mice was associated with ~10% lower Mr than B6129F2 mice during non-heated control experimentation and prior to heat exposure in the calorimeter. The hypothermic state of the TNFR KO genotype appeared to impart an advantage during heat exposure, in that these mice maintained a lower Tc for any given duration of heat exposure despite attaining the same Tc,Max as B6129F2 mice. These findings contradict a recent study in rats that failed to show an effect of the TNFα monoclonal antibody infliximab on hyperthermia during an acute heat exposure (28). There are several reasons that may account for these study discrepancies including species differences, the use of anesthetized rats versus conscious mice, different heat exposure times / intensities or different experimental approaches to inhibit TNF actions. Since Smith et al. (28) were unable to detect plasma TNFα in any of the rats at the end of heat exposure, it was difficult to discern if infliximab inhibited TNF actions in their model. Our use of TNFR KO mice overcame this experimental limitation, but the developmental neutralization of TNF signaling induced changes in resting Tc, which affected the heat stress response, thus limiting a direct comparison of study outcomes.

The circadian, behavioral and heat stress responses of TNFR KO mice are remarkably similar to those observed in hypothyroid rats exposed to the same types of control and heat stress conditions (31). That is, rats rendered hypothyroid following the injection of propylthiouracil (PTU) showed similar motor activity levels as controls, but developed a regulated state of hypothermia that was characterized by lower Tc than control rats during normal housing across a wide range of Ta (31). Similar to TNFR KO mice, PTU rats selected warmer temperatures in the gradient, but maintained a lower Tc than control rats despite these behavioral adjustments. Also, ~120 min heat stress reversed the hypothermic state of the PTU rats, but Tc remained lower than controls, similar to the responses observed in the calorimeter in the current study. Although
thyroid hormone levels would be expected to decrease during heat exposure as a compensatory mechanism to minimize heat production, hypothyroidism was purported by Yang and Gordon to induce compensatory increases in thyroid-stimulating hormone (TSH) that may have had downstream effects on hypothalamic pathways of Tc control. It is intriguing to speculate that TNF (or perhaps other cytokines, such as IL-1α) may modulate M_r via interactions with the hypothalamo-pituitary-thyroid axis in our model. In mice and rats, TNF injection decreased TSH release (20, 21), which is congruent with the hypothesis that neutralization of TNF signaling may have increased this hormone and induced regulated hypothermia through similar mechanisms as those identified with hypothyroidism. Unfortunately, we did not have adequate plasma volume to measure TSH or plasma thyroid hormone levels in the TNFR KO mice such that additional studies will be required to determine the mechanism by which TNF modulated mechanisms of Tc and M_r control in our model.

We were unable to detect changes in plasma TNFα at Tc,Max, RTB, or HD in B6129F2 mice, which replicates recent studies in C57BL/6J and C3H/HeJ mice heated to a higher Tc,Max of 42.7°C (7, 15). Conversely, TNFR KO mice showed high plasma TNFα levels at all sampling time points of recovery presumably due to the lack of TNFR negative feedback on synthesis of this cytokine. Since TNF only binds to cells that contain the TNFRI and II and we verified the absence of the receptors in the KO mice, it is unlikely that the high circulating TNF levels in the KO mice induced nonspecific effects on the responses observed in this study (22). However, sTNFRI and II were elevated in B6129F2 mice from Tc,Max through HD suggesting negative feedback of TNF actions were occurring at the cellular level and not readily apparent from circulating measures of the cytokine. As such, we propose that circulating sTNFRs are more sensitive biomarkers of TNF actions in heat stroke than the cytokine itself. Plasma cytokines
have never been measured *during* heat exposure so their actions in $T_c$ and metabolic control in this condition remain poorly understood. To our knowledge, IL-1 is the only other cytokine that has been implicated in $T_c$ control during heat exposure with IL-1RI KO mice requiring longer heat exposures to reach $T_{c,\text{Max}}$ than wild-type mice (11). Although increases in IL-1 / sIL-1Rs were not measured during heat exposure and these proteins did not change at $T_{c,\text{Max}}$, lower baseline levels of these IL-1 family members in the KO mice suggest interactions between TNF and IL-1 may modulate $T_c$ and/or $M_r$ under resting or heat exposure conditions. Future studies with greater temporal resolution of TNF and other cytokine release kinetics will be required to more accurately delineate the time course of cytokine actions, alone or in combination with one another during heat exposure and recovery in our model. It will also be important to validate our findings using short-term TNF inhibitors in multiple species to more precisely determine the actions of this cytokine on the hyperthermic and metabolic response to heat exposure and support extrapolation of our findings to other model systems that do not exhibit developmental alteration of the resting $T_c$ and metabolic state.

TNFR KO mice showed significantly lower hepatic SAA3 gene expression at HD compared to B6129F2 mice suggesting the APR was compromised in these animals during recovery. Previous heat stroke studies have shown increased circulating C-reactive protein and liver $\alpha_1$-acid glycoprotein mRNA expression during heat stroke recovery (4, 32); however, this is the first study to demonstrate an effect of heat stroke on liver SAA gene expression and implicate TNF in regulation of this response during recovery. IL-1, TNF and IL-6 are known inducers of SAA3 with additive and/or synergistic effects on transcriptional regulation of these genes (12). For example, TNF $\alpha/\beta$ as well as IL-6 KO mice showed attenuated (~50%) plasma SAA protein levels in response to the peripheral injection of lipopolysaccharide (a cell wall component of
gram negative bacteria). However, this response was completely abrogated in TNFα/β/IL-6 KO mice suggesting the concerted actions of TNF and IL-6 are essential for SAA production following this systemic inflammatory insult (3, 8). Interestingly, SAA protein changes in TNFα/β/IL-6 KO mice occurred in the absence of differences in liver SAA mRNA expression indicating posttranscriptional modification of this response (3). Unfortunately, limited plasma volume prevented measurement of SAA proteins in B6129F2 and TNFR KO mice, but will be an important measurement in future studies since these are considered sensitive biomarkers of inflammation severity. The IL-6-STAT3 pathway is considered an important regulator of liver SAA transcription. For example, hepatic STAT3 conditional mutant mice showed complete abrogation of SAA1, 2, and 3 mRNA expression in response to IL-6 (1). Although plasma IL-6 was similar between genotypes in our study, TNFR KO mice showed a more robust sIL-6R response, which may have buffered IL-6 effects on downstream STAT3 phosphorylation and SAA3 gene expression in the liver. Specifically, IL-6 would be expected to form complexes with the additional sIL-6R in the plasma of the KO mice, which would inhibit binding to trans-membrane receptors (26). However, we cannot discount the possibility that other cytokine-mediated mechanisms modulate APP gene expression during heat stroke recovery in our model. We recently showed that liver IL-1α and β gene expression was more rapidly induced in B6129F2 than TNFR KO mice during recovery (25), which occurred prior to SAA3 gene expression changes. Given the complex interactions of IL-1, TNF and IL-6 in regulation of the APR, future studies will be required to determine the mechanisms of SAA gene regulation during heat stroke recovery in mice. It is important to note that the differential liver p-STAT3 and SAA3 responses between genotypes were not associated with differences in HSP70 protein expression in this organ. HSP70 is considered a sensitive biomarker of organ stress that is
induced in response to many physiological perturbations including heat, ischemia and oxidative
damage (13). All of these factors were presumably operative in our model, but similar liver
HSP70 protein profiles suggest the attenuated APP response was a downstream effect of the
absence of TNF signaling and not merely a consequence of differences in thermal stress / injury
to this organ. Since we did not measure heat stroke responses beyond ~3h of recovery and
plasma IL-1β and IL-6 were shown to peak beyond this time point in a conscious mouse model
(7), the effect of TNF neutralization on the APP and multi-organ failure during the latter stages
of recovery will need to be determined in future studies.

Perspectives/Significance

Cytokines, such as IL-1, IL-6 and TNF have been implicated as adverse mediators of heat
stroke despite limited understanding of their actions on the thermoregulatory, metabolic and
inflammatory processes that affect outcome. Emerging evidence suggests that classification of
TNF and IL-6 as “pro-inflammatory” cytokines inaccurately represents the immunomodulatory
actions of these cytokines during heat stroke recovery (or perhaps other disease states). Our
findings suggest that TNF inhibition may be beneficial for thermoregulatory / metabolic control
during heat exposure, but the downside of such a therapeutic intervention is the potential
immunosuppressive effects on the APR during recovery. Of course, our results need to be
interpreted in the context of chronic developmental neutralization of TNF signaling in the KO
mice, which induced resting Tc and cytokine changes that limit extrapolation to other model
systems. Interestingly, chronic use of TNFα inhibitors to treat rheumatoid arthritis and other
inflammatory conditions is often associated with immunosuppression and increased risk of
infection, further supporting the hypothesis that TNF is important for immune health. Therefore,
it will be necessary to validate our findings from TNFR KO mice using acute and chronic administration of traditional TNFα inhibitors to determine the potential benefit of this therapeutic treatment on heat stroke susceptibility and outcome.
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The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

In conducting the research described in this report, the investigators adhered to the “Guide for Care and Use of Laboratory Animals” as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.
Figure 1. Experimental protocol. Three groups of mice were used. (A) Circadian and behavioral thermoregulatory responses of B6129F2 and TNFR KO mice. Approx. 1 week following transmitter implantation, circadian core temperature ($T_c$) and activity (0600-0559h) were measured at $25 \pm 2^\circ C$ in a cage with bedding, food and water ad libitum. The following day, mice were placed into the temperature gradient to acclimate to the runway at ambient temperature ($T_a$) $\sim 25^\circ C$ for $\geq 16$ hours. $T_c$ and selected temperature ($T_s$) were measured the following day (0600-0559h) at runway temperatures $\sim 18-40^\circ C$. Sample size was N=10 mice/genotype. (B) $T_c$ and metabolic rate ($M_r$) of B6129F2 and TNFR KO mice during heat exposure in an indirect calorimeter. Approx. 1 week following radiotelemetry implantation, mice were placed into the indirect calorimeter to acclimate to this environment at $T_a \sim 25^\circ C$ for $\geq 16$ hours. Starting at $\sim 0800-1000h$ the following day, $T_c$ and $M_r$ of non-heated control mice were measured for $\sim 4$ hours at $T_a \sim 25^\circ C$. Mice were removed from the calorimeter to recover for $\geq 1$ week and then re-acclimated to the indirect calorimeter at $\sim 25^\circ C$ for $\geq 16$ hours. Starting at $\sim 0800-1000h$ the following day, mice were exposed to $39.5 \pm 0.5^\circ C$ in the indirect calorimeter until $T_{c,\text{Max}}$ of 42.4°C was reached. $T_c$ and $M_r$ were monitored throughout non-heated control and heat stroke experimentation. Sample size was N=8 mice/genotype, with each mouse exposed to the non-heated control and heat stroke condition, in that order. (C) Plasma cytokine, soluble cytokine receptor and liver acute phase protein expression of B6129F2 and TNFR KO mice. Approx. 1 week following transmitter implantation, B6129F2 and TNFR KO mice were placed into a floor-standing incubator at $T_a$ of 25°C to acclimate to this environment for $\geq 16$ hours. Starting the following day at $\sim 0800-1000h$, heat stroke mice were sacrificed at time 0 (baseline; Group I) or exposed to $T_a$ of 39.5 $\pm$ 0.2°C until $T_{c,\text{Max}}$ of 42.4°C was reached. Blood and liver were collected at $T_{c,\text{Max}}$ (Group II), return-to-baseline (Group III), or hypothermia depth (Group IV). Non-heated control mice were exposed to $T_a$ of 25 $\pm$ 2°C with sampling time points time-matched to heat stroke mice. $T_c$ was monitored throughout experimentation. Sample size was N=6-8 mice/time point/genotype. Gray boxes represent time of data collection and analysis.

Figure 2. (A) Circadian core temperature and (B) motor activity profiles of B6129F2 and TNFR KO mice during normal housing at $T_a$ of 25°C (0600-0559h). Sample size was 10 mice/genotype. Data are 1-h averages. Black horizontal bar represents lights-off (active) period during a 12:12h L:D cycle. *Significant difference between genotypes at P<0.001.

Figure 3. (A) Circadian (0600-0559h) core temperature and selected temperature (B) profiles of B6129F2 and TNFR KO mice in a temperature gradient (18-40°C). Sample size was 10 mice/group. Data are 1-h averages. Black horizontal bar represents lights-off (active) period. *Significant difference between genotypes at P<0.001.

Figure 4. Core temperature (A, C) and metabolic rate (B, D) of B6129F2 and TNFR KO mice during non-heated control (left side) and heat exposure conditions (right side; $T_{c,\text{Max}}=42.4^\circ C$) in an indirect calorimeter. Sample size was 8 mice/genotype; each mouse served as its own control with $\geq 1$ week between experiments. Arrow at $time 0$ represents the start of heat exposure ($T_a=39.5 \pm 0.5^\circ C$). Data are 10-min averages. Data are shown for $\sim 210$ min (B6129F2) and $\sim 270$
min (TNFR KO), which represents the time point at which the first mouse of each group reached $T_{c,\text{Max}}$. *Significant difference between genotypes at $P<0.05$.

Figure 5. Representative core temperature curves of non-heated control and heat stroke B6129F2 and TNFR KO mice. The time points of blood and liver collection are indicated on the graph with non-heated control mice time-matched to that of a heated mouse. Group descriptors are provided under Materials and Methods.

Figure 6. Plasma (A) IL-1$\alpha$, (B) IL-1$\beta$, (C) IL-6, (D) IL-10 and (E) TNF$\alpha$ concentrations of B6129F2 and TNFR KO mice at baseline and during heat stroke recovery. Sample size was 6-8 mice/group. *$P<0.05$ vs. time-matched controls of same genotype; †$P<0.05$ vs. $T_{c,\text{Max}}$; §$P<0.05$ vs. return-to-baseline. Lines between bars represent significant difference between genotypes at $P<0.05$.

Figure 7. Plasma (A) sgp130, (B) sIL-1RI, (C) sIL-1RII, (D) sIL-6R, (E) sTNFRI and (F), sTNFRII concentrations of B6129F2 and TNFR KO mice at baseline and during heat stroke recovery. Sample size was 6-8 mice/group. *$P<0.05$ vs. time-matched controls of same genotype; †$P<0.05$ vs. $T_{c,\text{Max}}$; §$P<0.05$ vs. return-to-baseline. Lines between bars represent significant difference between genotypes at $P<0.05$. $T_{c,\text{Max}}$, maximum core temperature; RTB, return-to-baseline; HD, hypothermia depth; N.D., not detected.

Figure 8. (A) Representative Western blot and (B) liver HSP70 protein levels normalized to GAPDH protein levels (integrated intensities) of B6129F2 and TNFR KO mice at baseline and during heat stroke recovery. Sample size was 6-8 mice/group. *$P<0.05$ vs. time-matched controls of same genotype; †$P<0.05$ vs. $T_{c,\text{Max}}$; §$P<0.05$ vs. return-to-baseline. $T_{c,\text{Max}}$, maximum core temperature; RTB, return-to-baseline; HD, hypothermia depth.

Figure 9. (A) Liver phosphorylated STAT3 concentrations and (B) SAA3 mRNA fold-change of B6129F2 and TNFR KO mice at baseline and during heat stroke recovery. SAA3 levels were measured at hypothermia depth (HD) only. Sample size was 6-8 mice/group. *$P<0.05$ vs. time-matched controls of same genotype; †$P<0.05$ vs. $T_{c,\text{Max}}$; §$P<0.05$ vs. return-to-baseline. Lines between bars represent significant difference between genotypes at $P<0.05$. $T_{c,\text{Max}}$, maximum core temperature, RTB, return-to-baseline; HD, hypothermia depth.


Gene expression was compared to control of same genotype.

Temperature during heat exposure: RTD, return-to-baseline; T<sub>min</sub>, minimum core temperature during cooling.

Values are mean ± SD; sample sizes are indicated in parentheses. *p<0.05 between indicated genotypes or control. Data are representative of results presented. Heat exposure data represent all mice with animal numbers listed in parentheses. Data were combined for ease of presentation. Heat exposure data represent all mice with animal numbers listed in parentheses. Data were combined for ease of presentation.

<table>
<thead>
<tr>
<th>Time to Return to Baseline (min)</th>
<th>Recovery</th>
<th>Heat Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal Load (% of Mice)</td>
<td></td>
<td></td>
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<tr>
<td>Time to Return (min)</td>
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</tr>
</tbody>
</table>

**Table 1.** Thermoregulatory Responses of B6129F<sup>2</sup> and TNPFR KO mouse during heat exposure and recovery.
Experimental Protocol

A. Experiment 1: Circadian and behavioral thermoregulatory profiles of B6129F2 and TNFR KO mice

<table>
<thead>
<tr>
<th>Transmitter implantation</th>
<th>0600</th>
<th>Mice placed into gradient</th>
<th>Recovery</th>
<th>Circadian $T_e$ and activity profiles</th>
<th>$T_r$ = 25±2°C</th>
<th>Acclimation</th>
<th>Runway Temperature ~25°C</th>
<th>0600</th>
<th>Behavioral profiles</th>
<th>Runway Temperature ~18–40°C</th>
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<tbody>
<tr>
<td>$T_e$ = 25±2°C</td>
<td>24 hours</td>
<td>≥16 hours</td>
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<tr>
<td>$T_e$ and $T_r$ monitored at 1-min intervals</td>
<td>T_e and T_r monitored at 1-min intervals</td>
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</tbody>
</table>

N=10 mice/genotype

B. Experiment 2: $T_e$ and $M_r$ of B6129F2 and TNFR KO mice during heat exposure in an indirect calorimeter

<table>
<thead>
<tr>
<th>Control Experiment</th>
<th>Transmitter implantation</th>
<th>Mice placed into calorimeter</th>
<th>Time 0 ~0800-1000</th>
<th>$M_r$ and $T_e$ monitored at 1-min intervals</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery</td>
<td>$T_e$ = 25±2°C</td>
<td>$T_e$ = 25±2°C</td>
<td>$T_r$ = 25±2°C</td>
<td>$T_e$ = 25±2°C</td>
<td>Recovery</td>
</tr>
<tr>
<td>≥1 week</td>
<td>≥16 hours</td>
<td>~4 hours</td>
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<td>≥1 week</td>
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<table>
<thead>
<tr>
<th>Heat Experiment</th>
<th>Mice placed into calorimeter</th>
<th>Time 0 ~0800-1000</th>
<th>$T_{e,Max}$ = 42.4°C</th>
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<tr>
<td>$T_e$ = 25±2°C</td>
<td>$T_r$ = 39.5±0.5°C</td>
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<tr>
<td>≥16 hours</td>
<td>~4 hours</td>
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</table>

N=8 mice/genotype

Same mice tested under control and heat conditions, in that order

C. Experiment 3: Plasma cytokine, soluble cytokine receptor and liver acute phase protein expression of B6129F2 and TNFR KO mice during heat stroke recovery

<table>
<thead>
<tr>
<th>Heat Experiment</th>
<th>Transmitter implantation</th>
<th>Mice placed into heating chamber</th>
<th>Recovery</th>
<th>Acclimation</th>
<th>(I) Sac Time 0 (Baseline) ~0800-1000</th>
<th>(II) Sac $T_{e,max}$ = 42.4°C</th>
<th>(III) Sac Return to Baseline</th>
<th>(IV) Sac Hypothermia Depth</th>
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<td>≥1 week</td>
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<td></td>
<td>$T_r$ = 25±2°C</td>
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</tbody>
</table>

N=6-8 mice/genotype at each time point with samples from control mice time-matched to heated mice

$T_e$ monitored at 1-min intervals
Figure 2
Leon et al.

A

Core Temperature (°C)

B6129F2

TNFR KO

38.0

37.0

36.0

LIGHTS OFF

6:00  8:00  10:00  12:00  14:00  16:00  18:00  20:00  22:00  00:00  2:00  4:00

B

Activity (Counts)

30.0

20.0

10.0

0.0

LIGHTS OFF

6:00  8:00  10:00  12:00  14:00  16:00  18:00  20:00  22:00  00:00  2:00  4:00

Circadian Time (Hour Averages)
Figure 3
Leon et al.
Figure 7
Figure 8
Leon et al.
Figure 9
Leon et al.

A

B6129F₂

TNFR KO

P-STAT3 (MFI)

0 10 20 30 40 50 60 70 80 90 100

Baseline  Tc,Max  RTB  HD

B

SAA3 (Fold-Change)

0 1 2 3 4 5

Hypothermia Depth