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Attenuated thermoregulatory, metabolic, and liver acute phase protein response to heat stroke in TNF receptor knockout mice

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HEAT STROKE is characterized by severe hyperthermia and development of a systemic inflammatory response syndrome (SIRS) that often progresses to multiorgan 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 [sTNF-RII, 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 core temperature (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 Tc, gut damage, and survival of rats after ~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 Tc,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 Tc,Max and more rapid cooling (a protective Tc response), but showed a trend toward increased mortality compared with 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 thermoregula-
ory, 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 whether the effects of TNF neutralization on Tm responses was specific to heat stress or represented a general effect on all thermoregulatory processes. Metabolic rate (M) was measured due to reliance of mice on this autonomic mechanism for Tm 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, during heat exposure. M and Tm responses of B6129F2 and TNFR KO mice were compared during heat exposure to the same Tc, 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 (B6129SF-Tnfrsf1a1tm1mx Tnfrsf1b1tm1mx/J; Jackson Laboratories, Bar Harbor, ME) mice weighing 26.9 ± 1.0 g were used (n = 143 mice). Mice were individually housed in Nalgene polycarbonate cages (11.5 in. × 7.5 in. × 5 in.) fitted with Hepa-filter cage tops and wood chip bedding (Pro-Chip, PWI). Rodent laboratory chow (Harlan Teklad, LM-485, Madison, WI) and water were provided ad libitum under standard laboratory conditions (25°C; 12:12 h light-dark (L/D) cycle, lights on at 0600 h). Environmental enrichment was provided in each cage as a Nalgene Mouse House (Nalge Nunc, Rochester, NY) with a maple wood product (product no. W0002, Bio-Serv, Frenchtown, NJ) that contained 25% bedding (Pro-Chip, PWI). Rodent laboratory chow (Harlan Teklad, LM-485, Madison, WI) and water were provided ad libitum under standard laboratory conditions (25°C; 12:12 h light-dark (L/D) cycle, lights on at 0600 h). Environmental enrichment was provided in each cage as a Nalgene Mouse House (Nalge Nunc, Rochester, NY) with a maple wood product (product no. 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 before 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 (3.5 g, volume 1.75 ml; model TA10TA-F20, Data Sciences International, St. Paul, MN). The transmitter emitted a unique frequency (Hz) that was proportional to Tm and detected by an antenna under the animal’s cage; frequency values were transferred to a peripheral PC and converted to Tm 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 no. F6698, F6699, F6700, and F6701; Bio-Serv, Frenchtown, NJ) that contained 25 μg of indomethacin (Sigma no. I-8280, St. Louis, MO). Pellets were placed onto the cage floor for voluntary consumption ~1 h before surgery and at 0800 h on days 1, 2, and 3 of recovery. Consumption of the pellet was visually confirmed. Surgical recovery was achieved at ~1 wk, as defined by a return to presurgical body weight (minus transmitter weight), reestablishment of normal food and water consumption, and stable circadian Tm and activity, as previously described (18).

Heat stress protocol. The heat stress protocol has been described in detail elsewhere (16). Briefly, ~24 h before heat exposure, mice in their home cages with food, water, and bedding were placed into a floor-standing incubator (model 3950, Therna Forma, Marietta, OH) at the normal housing temperature of 25 ± 2°C to acclimate to the fan noises. Cage filter tops were removed to facilitate air circulation. The following day between ~0800 and 1000 h, mice with baseline Tm < 36.0°C were weighed, food, water, and enrichment products were removed from the cage, and the incubator ambient temperature (Tb) was increased to 39.5 ± 0.2°C (required ~1 h to reach this Tb). Mice were anesthetized with isoflurane, and the abdominal fur was shaved and scrubbed with a 10% Povidone-iodine solution (Betadine solution, Purdue Frederick, Stamford, CT) followed by 70% isopropyl alcohol. Each radiotelemetry device was disinfected by presoaking for 1 h in cold sterilant (Actril, Minnetech, Minneapolis, MN) followed by three rinses in sterile saline (0.9%). An ~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 nonabsorbable suture (6-0 Prolene, P-1 Reverse Cutting, Ethicon, Somerville, NJ) in interrupted and continuous subcuticular patterns, respectively. Immediately after surgery, each mouse was placed into a clean cage with ad libitum food and water and returned to the animal room for undisturbed recovery. Tm and activity were continuously monitored at 1-min intervals using the Vitalview (G2 emitter; Mini Mitter) or Dataquest Data Acquisition system (TA10TA-F20 model, Data Sciences International). Each transmitter emitted a unique frequency (Hz) that was proportional to Tm and detected by an antenna under the animal’s cage; frequency values were transferred to a peripheral PC and converted to Tm 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.

For all other experiments, mice were implanted with a battery-powered radiotelemetry transmitter (1.1 g, volume 0.52 ml; model G2 Emitter, Mini Mitter; Bend, OR). Both transmitters allowed remote measurement of Tb (± 0.1°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, Stamford, CT) followed by 70% isopropyl alcohol. Each radiotelemetry device was disinfected by presoaking for 1 h in cold sterilant (Actril, Minnetech, Minneapolis, MN) followed by three rinses in sterile saline (0.9%). An ~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 nonabsorbable suture (6-0 Prolene, P-1 Reverse Cutting, Ethicon, Somerville, NJ) in interrupted and continuous subcuticular patterns, respectively. Immediately after surgery, each mouse was placed into a clean cage with ad libitum food and water and returned to the animal room for undisturbed recovery. Tm and activity were continuously monitored at 1-min intervals using the Vitalview (G2 emitter; Mini Mitter) or Dataquest Data Acquisition system (TA10TA-F20 model, Data Sciences International). Each transmitter emitted a unique frequency (Hz) that was proportional to Tm and detected by an antenna under the animal’s cage; frequency values were transferred to a peripheral PC and converted to Tm 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.

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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 ± 2°C. Nonheated control mice were exposed to the same conditions at \( T_a \) of 25 ± 2°C with the timing of experimental procedures matched to that of a heat-exposed mouse. The experimental protocol for this study is depicted in Fig. 1 with details provided below.

### Experimental Protocol

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

<table>
<thead>
<tr>
<th>Transmitter implantation</th>
<th>Recovery</th>
<th>0600</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0600</td>
<td>0559</td>
</tr>
<tr>
<td></td>
<td>0559</td>
<td>END</td>
</tr>
</tbody>
</table>

- **Transmitter implantation:**
  - \( T_e=25±2°C \)
- **Recovery:**
  - \( T_e=25±2°C \)
- **Behavioral profiles:**
  - \( T_e=25±2°C \)

- **Acclimation**:
  - \( T_a=25±2°C \)
  - \( T_a=25±2°C \)

- **Time 0 (Baseline)**:
  - \( T_e=25±2°C \)
  - \( T_e=25±2°C \)

- **Runway Temperature**:
  - \( ~25°C \)
  - \( ~18-40°C \)

- **N=10 mice/genotype**

B **Experiment 2: \( T_c \) 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>Recovery</th>
<th>Acclimation</th>
<th>M_r and ( T_c ) monitored at 1-min intervals</th>
<th>Recovery</th>
<th>Return to home cage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0600</td>
<td>0559</td>
<td></td>
<td>0559</td>
<td>END</td>
</tr>
</tbody>
</table>

- **Transmitter implantation**:
  - \( T_e=25±2°C \)
  - \( T_e=25±2°C \)
  - \( T_e=25±2°C \)

- **Mice placed into calorimeter**:
  - \( T_e=25±2°C \)
  - \( T_e=25±2°C \)

- **Acclimation**:
  - \( T_a=25±2°C \)
  - \( T_a=39.5±0.5°C \)

- **Time 0 (Baseline)**:
  - \( T_a=25±2°C \)
  - \( T_a=25±2°C \)

- **Runway Temperature**:
  - \( ~25°C \)
  - \( ~18-40°C \)

- **N=8 mice/genotype**

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>(I) Sac</th>
<th>(II) Sac</th>
<th>(III) Sac</th>
<th>(IV) Sac</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time 0 (Baseline)</td>
<td>( T_{c,\text{Max}} )</td>
<td>Return to Baseline</td>
<td>Hypothermia Depth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>~0800-1000</td>
<td>~25°C</td>
<td>T_e=25±2°C</td>
<td>T_e=25±2°C</td>
</tr>
</tbody>
</table>

- **Transmitter implantation**:
  - \( T_e=25±2°C \)
  - \( T_e=25±2°C \)

- **Mice placed into heating chamber**:
  - \( T_e=25±2°C \)
  - \( T_e=25±2°C \)

- **Acclimation**:
  - \( T_a=25±2°C \)
  - \( T_a=25±2°C \)

- **Time 0 (Baseline)**:
  - \( T_a=39.5±0.2°C \)
  - \( T_a=25±2°C \)

- **Runway Temperature**:
  - \( ~18-40°C \)

- **N=6-8 mice/genotype at each time point**

- **N=6-8 mice/genotype**

- **Samples from control mice time-matched to heated mice**

N=8 mice/genotype

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

Acclimation ≥16 hours

Runway Temperature ~25°C

Ta=25±2°C

~4 hours

Mr and \( T_c \) monitored at 1-min intervals

≥24 hours

≥24 hours

Return to home cage

Ta=25±2°C

~3 hours

~30 min

Recovery

Ta=25±2°C

T,e monitored at 1-min intervals

# AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00127.2013 • www.ajpregu.org
Experiment 1: circadian and behavioral thermoregulatory profiles of B6129F2 and TNFR KO mice. After ≥1 wk recovery from transmitter implantation, circadian Tc and activity profiles of B6129F2 and TNFR KO mice (n = 10 mice/genotype) were examined under normal housing conditions (0600 to 0559 h; Fig. 1A). Mice remained in their original cage throughout experimentation with Tc and activity recorded at 1-min intervals. After circadian Tc and activity profiles were assessed, mice were placed into a temperature gradient to compare behavioral thermoregulatory responses between strains. Each mouse was placed into the temperature gradient ≥16 h before 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 (0600–0559 h; Fig. 1A). The thermal gradient has been described in detail elsewhere (9). Briefly, the gradient runway was constructed of a series of copper bars cooler or heated by water circulating through copper tubing that encompassed opposite ends of the runway. Runway temperature ranged from ~18 to 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 (Tc) 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 Tc. Three equally spaced radiotelemetry wand receivers (model RLA-3000, Data Sciences) 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. After recovery from transmitter surgery (≥1 wk), mice were placed into the calorimeter chamber at Tc of 25°C with food and water to acclimate (42.4°C; (14, 16)]. After control experimentation, mice were allowed to recover for ~1 wk, mice were placed into the calorimeter chamber at Ta of 25°C with food and water to acclimate (42.4°C; (14, 16)]. After control experimentation, mice were allowed to recover for ~7 days, and then reacclimated to the calorimeter chamber for circadian and behavioral thermoregulatory analyses. After recovery from transmitter implantation (≥1 wk), nonheated 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 Tc of 25°C (Fig. 1C). Starting between ~0800 and 1000 h the next day, mice were randomly assigned to the following groups and euthanized at each time point for blood and liver collection with nonheated controls matched to a heat stroke mouse at each time point (Fig. 1C): group I baseline (Tc < 36.0°C; immediately before heat stress); B6129F2, n = 10, TNFR KO, n = 8; group II) Tc,Max; B6129F2 nonheated control, n = 7; TNFR KO nonheated control, n = 6; B6129F2 heat, n = 7; TNFR KO heat, n = 6; group III) return-to-baseline (RTB; first Tc value <36.0°C during cooling); B6129F2 nonheated control, n = 8; TNFR KO nonheated control, n = 8; B6129F2 heat, n = 8; TNFR KO heat, n = 9; or group IV) hyperthermia depth (HD; lowest 1-min Tc value during recovery with 0.01°C/min cooling rate); B6129F2 nonheated control, n = 6; TNFR KO nonheated 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 gauge 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, ~2,800 rcf). After being exsanguinated, mice were infused with cold heparinized (10 U/ml) sterile saline and the liver was 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, and TNF-α.

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 previously described. After recovery from transmitter implantation (≥1 wk), nonheated 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 Tc of 25°C (Fig. 1C). Starting between ~0800 and 1000 h the next day, mice were randomly assigned to the following groups and euthanized at each time point for blood and liver collection with nonheated controls matched to a heat stroke mouse at each time point (Fig. 1C): group I baseline (Tc < 36.0°C; immediately before heat stress); B6129F2, n = 10, TNFR KO, n = 8; group II) Tc,Max; B6129F2 nonheated control, n = 7; TNFR KO nonheated control, n = 6; B6129F2 heat, n = 7; TNFR KO heat, n = 6; group III) return-to-baseline (RTB; first Tc value <36.0°C during cooling); B6129F2 nonheated control, n = 8; TNFR KO nonheated control, n = 8; B6129F2 heat, n = 8; TNFR KO heat, n = 9; or group IV) hyperthermia depth (HD; lowest 1-min Tc value during recovery with 0.01°C/min cooling rate); B6129F2 nonheated control, n = 6; TNFR KO nonheated control, n = 8; B6129F2 heat, n = 8; TNFR KO heat, n = 8. Tc was monitored at 1-min intervals throughout experimentation.

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Plasma protein measurements. Mouse custom plex kits were used to analyze duplicate plasma samples for IL-1α, IL-1β, IL-6, IL-10, and TNF-α.
TNFR mice and heat stroke

Data analysis. Tc, activity, Tm, plasma cytokine, soluble cytokine receptor and liver HSP70 protein data are means ± SE. APP gene expression values are presented as mean fold change without SE. p-STAT3 data are presented as MFI ± SE. Tc and activity are presented as 1-h averages (circadian and behavioral thermoregulatory responses) or 10-min averages (indirect calorimeter experiment only). M was determined at 2- and 5-min intervals and averaged into 10-min bins for presentation. Nonheated 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, nonheated control values are graphically presented for baseline only. Time to Tc,max, thermal load, cooling time to baseline, time to HD, and SAA3 fold change were analyzed using 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, San Jose, CA).

Fig. 3. Circadian (0600–0559 h) core temperature (A) 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.
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 (Fig. 2, A and B). TNFR KO mice maintained ~0.4°C lower daytime Tc than B6129F2 mice from 0700 to 1600 h (35.9 ± 0.1°C vs. 36.2 ± 0.1°C, respectively; ANOVA, P < 0.001; Fig. 2A), whereas activity levels were similar between genotypes during this time (5.0 ± 1.0 vs. 5.3 ± 1.1, respectively; Fig. 2B). Nighttime Tc and activity were similar between genotypes with a peak from 1800 to 2200 h followed by a gradual return to baseline levels (Fig. 2, A and B).

The circadian rhythm displayed by B6129F2 and TNFR KO mice in the temperature gradient consisted of warm daytime Tc when Tc was low and cooler nighttime Tc when Tc was elevated (Fig. 3, A and B). TNFR KO mice maintained ~0.4°C lower daytime Tc than B6129F2 mice from 0600 to 1500 h (35.9 ± 0.0°C vs. 36.3 ± 0.2°C, respectively; ANOVA, P < 0.001) despite residing at ~2°C warmer Tc during this time period (0600–1300 h: 32.0 ± 0.2°C vs. 30.4 ± 0.6°C, respectively; ANOVA, P < 0.001; Fig. 3, A and B). Tc of TNFR KO mice was higher than B6129F2 mice from 2100–0100 h, but Tc did not differ between genotypes during this time (Fig. 3, A 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 nonheated control (Fig. 4, A and B) and heat stroke experimentation (Fig. 4, 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. Before 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; Fig. 4). Weighing and handling at time 0 induced ~2°C stress hyperthermia that was associated with a robust increase in M, from ~0 to 60 min that did not differ between genotypes during control (Fig. 4, A and B) or heat stroke experimentation (Fig. 4, C and D). During control experimentation, Tc and Mr of B6129F2 mice were 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 (Fig. 4, A and B). From ~70 to 210 min of heat exposure, TNFR KO mice maintained ~0.8°C lower Tc and ~15% lower M, than B6129F2 mice (ANOVA, P < 0.001; Fig. 4, C 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 with 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 euthanized at baseline, Tc,Max, RTB, or HD. Nonheated 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 Tc,Max (ANOVA, P = 0.007; Table 1). The increased heat exposure time of TNFR KO mice was associated with significantly greater dehydration than B6129F2 mice (11.8 ± 0.4 vs. 10.0 ± 0.5%, respectively; ANOVA, P < 0.001; Table 1), whereas thermal load was
Table 1. Thermoregulatory responses of B6129F2 and TNFR KO mice during heat exposure and recovery

<table>
<thead>
<tr>
<th></th>
<th>B6129F2 Control (n = 21)</th>
<th>B6129F2 Heat (n = 23)</th>
<th>TNFR KO Control (n = 22)</th>
<th>TNFR KO Heat (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydration, %</td>
<td>2.5 ± 0.2</td>
<td>10.0 ± 0.5**†</td>
<td>2.3 ± 0.2</td>
<td>11.8 ± 0.4**†</td>
</tr>
<tr>
<td>Time to Tc,Max, min</td>
<td>240 ± 8</td>
<td>2243 ± 11</td>
<td>240.6 ± 9</td>
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<tr>
<td>Recovery</td>
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<tr>
<td>Cooling time to baseline, Min</td>
<td>35 ± 2</td>
<td>31 ± 2*</td>
<td></td>
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</tr>
<tr>
<td>Time to hypothermia depth, min</td>
<td>188 ± 8</td>
<td>156 ± 11*</td>
<td></td>
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</tr>
<tr>
<td>Hypothermia depth, °C</td>
<td>36.1 ± 0.2†</td>
<td>30.8 ± 0.2†</td>
<td>35.6 ± 0.2†</td>
<td>30.6 ± 0.2†</td>
</tr>
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Values are means ± SE; n, sample sizes. Mice of same genotype and treatment showed similar core temperature responses and data were combined for ease of presentation. Heat exposure data represent all mice with animal numbers indicated in parentheses. Recovery data represent mice that were euthanized at return-to-baseline (RTB) or hypothermia depth. TNFR, tumor necrosis factor receptor; KO, knockout mice; Tc,Max, maximum core temperature during heat exposure; Tc,Min, minimum core temperature during cooling. *P < 0.05 between genotypes; †P < 0.05 compared with control of same genotype.

Figure 5 shows the sampling time points for blood and liver collection relative to the Tc responses displayed by each genotype during heat exposure and recovery.

Before heat exposure (baseline, time 0), TNFR KO mice showed significantly lower plasma IL-1α levels than B6129F2 mice (Fig. 6A), whereas baseline levels of plasma IL-1β (Fig. 6B), IL-6 (Fig. 6C), and IL-10 (Fig. 6D) were similar between genotypes. On the other hand, the plasma baseline level of TNFα was significantly higher in TNFR KO than B6129F2 mice (2,304.5 ± 350.7 vs. 409.3 ± 8.3 pg/ml; ANOVA, P < 0.001; Table 1). Plasma IL-1α and IL-1β did not change during heat stroke recovery in either genotype (Fig. 6, A and B). Plasma IL-6 was not increased in either genotype until HD with approximately two-to threefold increase above nonheated controls in both genotypes (ANOVA, P < 0.05; Fig. 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 vs. 500.5 ± 129.2 pg/ml; ANOVA, P = 0.016; Fig. 6D). TNFR KO mice maintained significantly higher plasma TNFα levels compared with B6129F2 mice throughout recovery, but heat stroke had no effect on this cytokine (ANOVA, P < 0.001; Fig. 6E).

Plasma sgp130 (Fig. 7A) and sIL-1RII (Fig. 7C) were similar between genotypes at all sampling time points. TNFR KO mice showed significantly lower sIL-1RII levels at baseline compared with B6129F2 mice (2,950 ± 151 vs. 5,140 ± 862 pg/ml; ANOVA, P = 0.031), but this soluble cytokine receptor did not change in either genotype during heat stroke recovery (Fig. 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; Fig. 7D). B6129F2 mice showed approximately fourfold higher sTNFRII than I levels at baseline (8,777 ± 769 vs. 2,190 ± 59 pg/ml, respectively; Fig. 7, E and F). B6129F2 mice showed increased plasma sTNFRI and II levels compared with nonheated controls from Tc,Max to HD (ANOVA, P < 0.001), whereas the sTNFRs were undetectable at all time points in the TNFR KO mice (Fig. 7, E 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 (Fig. 8, A 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 with the earlier time points of recovery (ANOVA, P < 0.05; Fig. 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; Fig. 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; Fig. 9A). SAA1, SAA4, Hp, FgA, FgB, and FgG gene expression were 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 nonheated controls at HD, which was more pronounced in B6129F2 compared with TNFR KO mice (3.8- vs. 2.7-fold, respectively; ANOVA, P = 0.009; Fig. 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, M, 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 Ta 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-1R, and Mr were lower in the KO compared with 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 with B6129F2 mice, suggesting IL-6 regulation of the APR may be compromised in these animals.

During housing at Ta 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 whether the lower Tc of TNFR KO mice was due to a metabolic deficiency or a decrease in the temperature set point (i.e., an effect on central nervous system mechanisms of Tc control), we examined behavioral responses in a gradient that ranged in temperature from ~18 to 40°C. If the lower Tc 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 Tc. This hypothesis was based on the observation that Ta of 39.5°C was sufficient to reverse the hypothermia of TNFR KO mice using our heat stress protocol (current study and Ref. 14). As shown in the gradient experiment, the set point for Tc 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 Tc of ~35.9°C in the gradient, which was virtually identical to that observed under normal housing conditions. The maintenance of similar daytime Tc under conditions in which a deficit in autonomic thermoeffectors could have been compensated for by thermo-regulatory behavior suggests that these mechanisms of Tc control were operating similarly to that observed under normal housing conditions. The maintenance of similar daytime Tc under conditions in which a deficit in autonomic thermoeffectors could have been compensated for by thermo-regulatory behavior suggests that these mechanisms of Tc 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 Tc response (i.e., due to an elevation in the temperature set point) that is associated with ~20% increase in Mr (17) The ability of TNFR KO mice to activate Tc and Mr to invoke a similar magnitude of fever as

Fig. 6. Plasma IL-1α (A), IL-1β (B), IL-6 (C), IL-10 (D), and TNFα (E) 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. Tc,Max; §P < 0.05 vs. return-to-baseline (RTB). Lines between bars represent significant difference between genotypes at P < 0.05.
B6129F2 mice indicates these mice are not metabolically deficient but simply residing at a lower baseline Tc than their wild-type counterparts.

The hypothermic state of the TNFR KO mice was associated with ~10% lower Mr than B6129F2 mice during nonheated control experimentation and before 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 Mr 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 Mr control in our model.

Fig. 7. Plasma sgp130 (A), sIL-1RI (B), sIL-1RII (C), sIL-6R (D), sTNFRI (E), and sTNFRII (F) 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. Tc,Max; §P < 0.05 vs. RTB. Lines between bars represent significant difference between genotypes at P < 0.05. N.D., not detected.
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 Tc and metabolic control in this condition remain poorly understood. To our knowledge, IL-1 is the only other cytokine that has been implicated in Tc control during heat exposure with IL-1RI KO mice requiring longer heat exposures to reach Tc,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 Tc,Max, lower baseline levels of these IL-1 family members in the KO mice suggest interactions between TNF and IL-1 may modulate Tc and/or Mr 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 Tc and metabolic state.

TNFR KO mice showed significantly lower hepatic SAA3 gene expression at HD compared with 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 α1-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 α/β as well as IL-6 KO mice showed attenuated (~50%) plasma SAA protein levels in response to the peripheral infection 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 transmembrane 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 before 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 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 ~3 h 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 multiorgan failure during the latter stages of recovery will need to be determined in future studies.

Perspectives and 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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AUTHOR CONTRIBUTIONS

Author contributions: L.R.L. and D.C.C. conception and design of research; L.R.L., S.M.D., and M.D.B. performed experiments; L.R.L., S.M.D., and M.D.B. analyzed data; L.R.L., S.M.D., M.R.-F., and D.C.C. interpreted results of experiments; L.R.L., prepared figures; L.R.L., M.R.-F., and D.C.C. drafted manuscript; L.R.L., M.R.-F., and D.C.C. edited and revised manuscript; L.R.L. approved final version of manuscript.

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