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<b>14. ABSTRACT</b> Interleukin-1 (IL-1) is thought to have a significant role in the pathophysiology of heat stroke (HS), although little is known regarding the actions or expression patterns of the IL-1 family. This study tested the hypotheses that following HS IL-1 family gene expression is dynamic, while loss of IL-1 signaling enhances recovery. IL-1 family expression was determined in plasma, spleen, and liver from C57BL/6J mice (n = 24 control, n = 20 HS) at maximum core temperature (Tc,Max), hypothermia, and 24 h post-HS (24 h). Soluble IL-1 receptor subtype I (sIL-1RI) protein expression peaked at 24 h (14,659.01 + 2,016.28 pg/ml, P < 0.05), while sIL-1RII peaked at hypothermia (19,099.30 + 1,177.07 pg/ml). IL-1 $\alpha$ gene expression in the spleen (ninefold) and liver (fourfold) along with IL-1RI (threefold spleen and fivefold liver) were maximal at hypothermia. Spleen IL-1 $\beta$ gene expression peaked at Tc,Max (fourfold) but at hypothermia (fourfold) in liver. Gene expression of the IL-1 family member IL-18 peaked (2.5-fold) at Tc,Max but was similar at all other time points. Subsequent studies revealed that despite accruing a greater heating area (298 + 16 vs 247 + 13°C-min, P < 0.05), IL-1RI knockout (KO) mice (n = 14) showed an attenuated hypothermia depth (28.5 + 0.2 vs 27.2 + 0.2°C, P < 0.05).					
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## Tissue and circulating expression of IL-1 family members following heat stroke

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**Helwig BG, Leon LR.** Tissue and circulating expression of IL-1 family members following heat stroke. *Physiol Genomics* 43: 1096–1104, 2011. First published August 9, 2011; doi:10.1152/physiolgenomics.00076.2011.—Interleukin-1 (IL-1) is thought to have a significant role in the pathophysiology of heat stroke (HS), although little is known regarding the actions or expression patterns of the IL-1 family. This study tested the hypotheses that following HS IL-1 family gene expression is dynamic, while loss of IL-1 signaling enhances recovery. IL-1 family expression was determined in plasma, spleen, and liver from C57BL/6J mice ( $n = 24$  control,  $n = 20$  HS) at maximum core temperature ( $T_{c,Max}$ ), hypothermia, and 24 h post-HS (24 h). Soluble IL-1 receptor subtype I (sIL-1RI) protein expression peaked at 24 h ( $14,659.01 \pm 2,016.28$  pg/ml,  $P < 0.05$ ), while sIL-1RII peaked at hypothermia ( $19,099.30 \pm 1,177.07$  pg/ml). IL-1 $\alpha$  gene expression in the spleen (ninefold) and liver (fourfold) along with IL-1RI (threefold spleen and fivefold liver) were maximal at hypothermia. Spleen IL-1 $\beta$  gene expression peaked at  $T_{c,Max}$  (fourfold) but at hypothermia (fourfold) in liver. Gene expression of the IL-1 family member IL-18 peaked (2.5-fold) at  $T_{c,Max}$  but was similar at all other time points. Subsequent studies revealed that despite accruing a greater heating area ( $298 \pm 16$  vs.  $247 \pm 13^\circ\text{C}\cdot\text{min}$ ,  $P < 0.05$ ), IL-1RI knockout (KO) mice ( $n = 14$ ) showed an attenuated hypothermia depth ( $28.5 \pm 0.2$  vs.  $27.3 \pm 0.5^\circ\text{C}$ ,  $P < 0.05$ ) and duration ( $675 \pm 82$  vs.  $1,283 \pm 390$  min,  $P < 0.05$ ) with a higher 24 h  $T_c$  ( $36.9$  vs.  $34.1^\circ\text{C}$ ,  $P < 0.05$ ) compared with C57BL/6J mice ( $n = 8$ ). The current results demonstrate that following HS IL-1 family gene expression is altered and IL-1RI KO mice display  $T_c$  responses consistent with a more rapid recovery.

liver; spleen; hypothermia; heat stress; cytokines

HEAT STROKE (HS) induced multiorgan failure (MOF) is thought to be due to the systemic inflammatory response syndrome (SIRS) that ensues following endotoxin leakage from ischemic gut barrier membranes (23, 24). Endotoxin is believed to stimulate proinflammatory cytokine release, which contributes to core temperature ( $T_c$ ) and inflammatory disturbances resulting in MOF and death. The mediators of HS morbidity/mortality have not been specifically identified, but results of correlation studies have implicated cytokines, including interleukin-1 (IL-1), as having important actions in the pathophysiology of HS.

Knowledge regarding the role of the IL-1 family in mediating HS morbidity/mortality is based solely on results of circulating plasma levels as no study has reported organ-specific cytokine changes as a result of HS. For example, high circulating levels of IL-1 $\alpha$  and IL-1 $\beta$  correlated with the severity of

hyperthermia at the time of clinical admission in HS patients (8, 9, 21, 36). In animal models, high central nervous system and/or circulating IL-1 $\beta$  levels were associated with hyperthermia at the time of HS collapse (32–34). Previously we reported that mice develop hypothermia ( $T_c \sim 29^\circ\text{C}$ ) within  $\sim 2$  h of HS recovery a  $T_c$  response that was correlated with high plasma IL-1 $\beta$  (but not IL-1 $\alpha$ ) levels (27).

The initial step in physiological responses directly mediated by IL-1 $\alpha$  and IL-1 $\beta$  require that the cytokine bind the IL-1 subtype I receptor (IL-1RI), the interaction then allows for recruitment of the IL-1 receptor accessory protein (IL-1RAcP) to the ligand/receptor complex resulting in formation of a functional signaling complex. IL-1 cytokines can also bind the IL-1 subtype II receptor (IL-1RII). However, the short intracellular tail of the IL-1RII prevents signal transduction following binding of the IL-1 ligand, thereby creating an antagonistic receptor that sequesters excess circulating IL-1. Tissue-bound receptors can also be truncated resulting in the formation of soluble forms of the receptors, sIL-1RI and sIL-1RII. Similar to the tissue bound IL-1RII receptor both soluble receptors act as antagonists of IL-1 and can influence physiological actions of the cytokines. Although overlooked to date, understanding the actions of tissue and soluble receptors provides critical insight regarding the regulation of IL-1 in the HS syndrome.

Similar to IL-1 $\alpha$  and IL-1 $\beta$ , IL-18, a recently identified member of the IL-1 family, is induced by lipopolysaccharide (LPS) injection (37) and has significant proinflammatory properties (14, 15). While injections of the cytokine do not directly induce a rise in  $T_c$ , administration of the cytokine attenuates IL-1 $\beta$ -induced elevations in  $T_c$  (17). These results suggest the cytokine may influence  $T_c$  responses following HS, although like IL-1 receptors, expression patterns of the cytokine in response to HS have yet to be determined.

Clearly, the time course of production, actions, and tissue-specific expression patterns of the IL-1 family members in the HS syndrome remain poorly understood. Insight regarding the expression patterns of IL-1 family members is especially important in organs such as the liver and spleen as both are major immune organs known to produce and respond to cytokines during inflammation. Furthermore, we have previously shown that the organs display a differential time course of injury in our mouse HS model (26, 27). Thus, understanding expression patterns of the IL-1 family members may provide important insight regarding the source and potential therapeutic benefit of targeted treatment strategies against select cytokines.

The purpose of this study was to examine IL-1 family expression at maximum  $T_c$  ( $T_{c,Max}$ ) and during recovery from HS in plasma, liver, and spleen of C57BL/6J mice. We hypothesized that HS recovery would be characterized by significant alterations in IL-1 family gene and protein expression in

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plasma, spleen, and liver samples. The results of gene expression studies revealed that HS was accompanied by dynamic changes in IL-1 cytokine expression. To further understand the physiological impact of changes in IL-1 expression during HS and recovery, we performed a follow-up set of experiments in which we investigated the role of IL-1 using our previously established mouse model of HS (28).

Current knowledge regarding the actions of IL-1 during HS have been derived from animal studies administering the IL-1 receptor antagonist (IL-1ra), a ligand that competes for the IL-1 binding site, thereby blocking IL-1-mediated actions (10, 11, 32, 33). However, use of the IL-1ra has yielded inconclusive results regarding the actions of IL-1 during and in recovery from HS. Alternatively, the IL-1 receptor subtype I knockout (IL-1RI KO) mouse lacks the IL-1R, the only known IL-1 receptor subtype that can directly propagate a signaling cascade. The use of a conscious IL-1RI KO mouse model of HS allows for normal heat dissipation methods while circumventing pharmacokinetic issues associated with injection of the receptor antagonist. Using the IL-1RI KO mouse a second goal of this study was to determine the effects of the loss of IL-1 signaling on  $T_c$  responses following HS in a conscious mouse model (28–30). We hypothesized that following HS IL-1RI KO mice would demonstrate  $T_c$  responses that are indicative of a more rapid recovery compared with C57BL/6J mice.

## MATERIALS AND METHODS

**Animals.** Specific pathogen-free male C57BL/6J mice and IL-1RI KO mice (Jackson Laboratories, Bar Harbor, ME) were used. For the C57BL/6J gene expression and plasma protein studies the control ( $n = 24$ ) and HS ( $n = 20$ ) groups weighed  $30.8 \pm 0.4$  g and  $31.5 \pm 0.5$  g, respectively (27). For the C57BL/6J ( $n = 8$ ) and IL-1RI KO ( $n = 14$ ) HS studies animals weighed  $29.1 \pm 0.6$  g (2–3 mo old) and  $32.1 \pm 0.6$  g (2 mo old), respectively.

Mice were individually housed in Nalgene polycarbonate cages ( $11.5 \times 7.5 \times 5$  in) fitted with HEPA-filter cage tops and wood chip bedding (Pro-Chip, PWI Industries). Rodent laboratory chow (LM-485; Harlan Teklad, Madison, WI) and water were provided ad libitum under standard laboratory conditions ( $25 \pm 2^\circ\text{C}$ , 12:12 h light-dark cycle, lights on at 0700). (27). In conducting research using animals, we adhered to the *Guide for the Care and Use of Laboratory Animals* (39) 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.

**Blood and tissue harvesting.** Blood, liver, and spleen were collected from C57BL/6J mice as described in detail previously (27). In brief, C57BL/6J mice were heat stressed and randomly assigned to the following groups for blood and tissue collection: Baseline ( $T_c < 36.0^\circ\text{C}$ ),  $T_{c,\text{Max}}$  ( $42.7^\circ\text{C}$ ), hypothermia (lowest  $T_c$  value with cooling rate of  $0.01^\circ\text{C}\cdot\text{min}$  during recovery), or 24 h following the start of heat exposure. Control groups were analyzed at each time point to account for any circadian influences on gene (tissue) and protein (plasma) expression. Sampling time of each control mouse was matched to that of a mouse in the corresponding HS group (27).

C57BL/6J mice were rapidly anesthetized ( $<1$  min) with isoflurane (5% in 100%  $\text{O}_2$ , flow rate =  $3.0$  l/min) and exsanguinated following thoracotomy and intracardiac puncture (heparinized 1 ml syringe, 23 gauge needle); blood was transferred to 1.5 ml heparinized microcentrifuge tubes and immediately placed onto ice until plasma could be separated by centrifugation ( $4^\circ\text{C}$ ; 5 min,  $\sim 3,500$  rpm). Plasma samples were aliquoted into  $\leq 150$   $\mu\text{l}$  volumes and stored at  $-80^\circ\text{C}$  until assayed. The spleen and liver were rapidly excised, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until analysis.

**Plasma cytokine measurements.** sIL-1RI and sIL-1RII concentrations were determined on duplicate plasma samples with kits specific for mouse soluble cytokine receptors (sensitivity 2 pg/ml) (27). Plasma samples were analyzed using the FlowMetrix System (Luminex, Austin, TX), which is a “Multiplexed Fluorescent Bead-Based Immunoassay.” Control groups at each time point revealed virtually identical sIL-1RI and sIL-1RII levels thus control groups were combined for ease of presentation. The immunoassay procedure used in this protocol detects the soluble receptor regardless of whether or not it is bound to it an IL-1 ligand. Thus the results presented represent total binding and are not segregated into bound and unbound fractions.

**RNA extraction.** Frozen liver and spleen samples were placed in TRI Reagent, homogenized, and RNA isolated using the RiboPure Kit (Ambion, Austin, TX). RNA concentration and quality were determined using a Nanodrop 8000 spectrophotometer (Nanodrop Products, Wilmington, DE). RNA concentration was calculated from the 260 nm reading. RNA purity for all samples used in real-time PCR analysis was  $\geq 1.8$  and  $\geq 1.95$  for the 260/230 and 260/280 nm ratios, respectively.

**Reverse transcription and real-time PCR.** RNA samples were reverse transcribed into cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Samples were reverse transcribed at  $37^\circ\text{C}$  for 2 h, and the resultant cDNA was used in real-time PCR experiments.

TaqMan Expression Assays containing 0.9  $\mu\text{M}$  of each primer and 0.25  $\mu\text{M}$  of a TaqMan MGB probe, comprising an FAM reporter dye at the 5'-end and a nonfluorescent quencher at the 3'-end, were combined with 50 ng cDNA and water in a final reaction volume of 20  $\mu\text{l}$ . Primers and probe sets for murine IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RI, IL-1RII, IL-1RAcP, IL-18,  $\beta$ -actin (Actb),  $\beta$ -glucuronidase (Gusb), phosphoglycerate kinase-1 (PGK-1), and 18s RNA were obtained from Applied Biosystems. Efficiency for the primer/probe sets was  $>90\%$  for all genes. To further 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. Amplification of PCR products was performed under the following conditions: 10 min at  $95^\circ\text{C}$  followed by 40 cycles consisting of  $95^\circ\text{C}$  for 15 s and 1 min at  $60^\circ\text{C}$ .

The threshold cycle ( $C_t$ ) for each gene was defined as the PCR cycle at which the emitted fluorescence signal was greater than the background level of fluorescence (18). Changes in target gene expression within spleen or liver were calculated as fold change relative to the controls at the same time point ( $T_{c,\text{Max}}$ , hypothermia, 24 h) using the  $2^{-\Delta\Delta C_t}$  method (35). Data were normalized, denoted as the  $\Delta C_t$ , by determining differences in  $C_t$  values between the target gene of interest and average of the internal control genes ( $\Delta C_t = C_t$  of target gene –  $C_t$  average of internal control genes). Mean normalized  $C_t$  values at baseline (control  $n = 6$ ),  $T_{c,\text{Max}}$  (control  $n = 7$ , heat  $n = 7$ ), hypothermia (control  $n = 5$ , heat  $n = 7$ ), and 24 h (control  $n = 6$ , heat  $n = 6$ ) were compared, and fold change was calculated if statistically significant changes in  $\Delta C_t$  existed. Fold change was calculated as  $2^{(\text{Savg}\Delta C_t - \text{CAvg}\Delta C_t)}$ , where  $\text{Savg}\Delta C_t - \text{CAvg}\Delta C_t$  was the difference between the sample (heated  $\Delta C_t$ ) and control (nonheated)  $\Delta C_t$  at a specific time point.

A panel of genes were screened using the  $2^{-\Delta C_t}$  method where  $-\Delta C_t = C_{t,\text{baseline}} - C_{t,\text{Time Point}}$  to identify appropriate internal control genes that did not change in response to heating (44). Target  $C_t$  values in liver samples were normalized to the expression levels of the internal control genes Gusb, PGK-1, and 18s, which were unaffected by the heating protocol. Within spleen tissue Actb was the only gene not significantly affected by the heating protocol and was used to normalize target gene  $C_t$  values in this tissue. Results of PCR data are presented as bar graphs of the mean fold change without error bars. This is necessary as cycle threshold is exponentially related to copy number, which is converted to fold change to derive linear comparisons. Thus  $2^{-\Delta\Delta C_t}$  estimates the error using  $\Delta\Delta C_t$  plus and  $\Delta\Delta C_t$  minus the SD (35).



***T<sub>c</sub> measurements.***  $T_c$  ( $\pm 0.1^\circ\text{C}$ ) was continuously monitored at 1 min intervals by radiotelemetry in conscious, free-moving C57BL/6J and IL-1RI KO mice using the Dataquest A.R.T. system (Data Sciences International, St. Paul, MN). Briefly, for transmitter implantation each mouse was anesthetized with isoflurane (4% induction, 2.5% maintenance in 100%  $\text{O}_2$ , flow rate = 3.0 l/min), and a temperature-sensitive transmitter (model TA10TA-F20) was implanted intra-abdominally using aseptic technique. Frequency of the emitted transmitter signal was proportional to  $T_c$ . An antenna placed under the cage of each animal received the emitted transmitter signal and converted it to  $T_c$  using predetermined calibration values. Each transmitter was magnetically activated  $\geq 24$  h prior to implantation to ensure stable  $T_c$  values prior to experimentation. Transmitters were calibrated before and after experimentation to ensure accuracy of  $T_c$  measurements. Ibuprofen analgesia (Children's Advil, Cold Formula, grape; Wyeth Healthcare, El Paso, TX) was provided in the drinking water 24 h prior to surgery (200  $\mu\text{g}/\text{ml}$ ) and by injection (30 mg/kg sc) immediately following transmitter implantation. Surgical recovery ( $\sim 14$  days) was required prior to heat stress experimentation, as defined a priori by a return to presurgical body weight and establishment of a robust, consistent circadian  $T_c$  rhythm, as previously described for this species (28, 31).

***Heat stress and heating calculations.*** The HS protocol has been described in detail elsewhere (28). Briefly, conscious unrestrained C57BL/6J and IL-1RI KO mice were exposed to an ambient temperature ( $T_a$ ) of  $39.5 \pm 0.2^\circ\text{C}$  in a floor-standing incubator, in the absence of food and water, until a maximum  $T_c$  ( $T_{c,\text{Max}}$ ) of  $42.7^\circ\text{C}$  was attained. Following removal from the heat, food and water were provided ad libitum during  $\sim 48$  h of undisturbed recovery at  $T_a$  of  $25 \pm 2^\circ\text{C}$ . This relatively cool recovery  $T_a$  (below the thermoneutral zone for this species, Ref. 20) was chosen as it is essential for HS recovery in mice (28). Control mice remained at  $T_a$  of  $25^\circ\text{C}$  in their original cage location and were not exposed to the incubator environment (31).

Various aspects of the  $T_c$  response during heat exposure and recovery were measured as previously described (27). Body weight (BW) was measured at baseline (immediately prior to the start of heat stress) and  $T_{c,\text{Max}}$  on a top-loading balance with an accuracy to  $\pm 0.1$  g. The difference in pre- and postheat stress BW was used as an estimation of percent dehydration using the following formula [(preheat stress BW – postheat stress BW)/preheat stress BW]  $\times 100$ . Time to  $T_{c,\text{Max}}$  (min) was calculated as the time from baseline (heat stress start at time 0) to  $T_{c,\text{Max}}$  ( $42.7^\circ\text{C}$ ). The time to  $T_{c,\text{Min}}$  (lowest  $T_c$  during recovery) was calculated from the time the animal was removed from the heat stress environment at  $T_{c,\text{Max}}$  until the lowest  $T_c$  value was attained in recovery. Heating area ( $^\circ\text{C}\cdot\text{min}$ ; area under the curve) was calculated as the  $\sum$  of the time intervals (min)  $\times 0.5$  ( $^\circ\text{C}$  above  $T_c = 39.5$  at the start of the interval +  $^\circ\text{C}$  above  $T_c = 39.5^\circ\text{C}$  at the end of the interval) until  $T_{c,\text{Max}}$  ( $42.7^\circ\text{C}$ ) was attained and used as an index of the heat stress incurred in the current study. The  $T_c = 39.5^\circ\text{C}$  was used in heating area calculations as  $T_c$  above this temperature allowed mice to radiate excess body heat to the environment. Hypothermia ( $^\circ\text{C}$ ) represents the lowest 1 h average  $T_c$  value observed during HS recovery. Hypothermia duration (min) was the total time that  $T_c$  was  $< 34.5^\circ\text{C}$  (represents the lowest circadian  $T_c$  value observed in an undisturbed C57BL/6J mouse in our laboratory; unpublished observation). Rewarming time (min) represents the time required to rewarm to  $\geq 34.5^\circ\text{C}$  from hypothermia. Previously we demonstrated that the inability to recover  $T_c$  from hypothermia by 765 min following initiation of the HS protocol was indicative of mortality (28). This time reference was used as a humane endpoint in the current study. The time points of 24 and 48 h are calculated as the elapsed time from the beginning of the HS protocol.

***Genotyping of IL-1RI KO animals.*** At time of tissue harvest  $\sim 1$  cm of tail was obtained from C57BL/6J and IL-1RI KO mice. 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 the IL-1RI (Operon, Huntsville, AL). Resultant PCR products were run on 1% agarose gels, and banding patterns were compared to confirm the absence of IL-1RI DNA.

***Statistical analysis.*** Data are presented as means  $\pm$  SE for  $T_c$  and plasma-soluble cytokine receptor data. Normalized Ct values within the same tissue (liver or spleen) and treatment (nonheated control or heat stress) were compared across time points with a one-way ANOVA. A one-way ANOVA comparing control animal (i.e., non-heated) normalized Ct values at each time point revealed no differences, and thus all controls were combined for ease of presentation. Fold change ranges were calculated and presented as mean fold change  $\pm$  SD. Plasma cytokine and  $T_c$  values between control and heat stress groups were compared by a one-way ANOVA. A two-way ANOVA was used to compare the  $T_c$  of C57BL/6J and IL-1RI KO mice during heat exposure and recovery with nonsurvivors included in these calculations up to  $T_{c,\text{Max}}$  only, a time-point to which all mice survived. Sample sizes of nonsurvivors were not large enough to statistically compare the  $T_c$  values of survivors. All statistics were performed with Sigma Plot 12.0 (Systat Software, San Jose, CA), and  $P < 0.05$  was considered significant.

## RESULTS

***Plasma sIL-1RI and sIL-1RII levels.*** sIL-1RI and sIL-1RII showed a differential time course of expression in C57BL/6J mice during 24 h of HS recovery. Plasma levels of sIL-1RI were not different from controls ( $2,890.6 \pm 101.7$  pg/ml) at  $T_{c,\text{Max}}$  ( $2,727.6 \pm 500.1$  pg/ml) or hypothermia ( $4,621.2 \pm 707.8$  pg/ml, Fig. 1A). At 24 h post-HS sIL-1RI levels ( $14,659.0 \pm 2,016.3$ ) were significantly elevated compared with all other time points (ANOVA,  $P < 0.001$ , Fig. 1A). Conversely, plasma sIL-1RII levels were significantly higher at hypothermia ( $19,099.3 \pm 1,177.1$  pg/ml) compared with all other time points ( $\sim 15,520$  pg/ml; ANOVA,  $P = 0.015$ ; Fig. 1B). Interestingly, plasma sIL-1RII levels were about four- to sixfold higher than plasma sIL-1RI levels at all time points except 24 h of recovery when they were similar (Fig. 1, A and B).

***Spleen and liver gene expression in C57BL/6J animals following HS.*** Control mice showed no difference in gene expression profiles at any time points and were combined into a single group for presentation purposes and arbitrarily assigned a value of 1 (dashed line, Fig. 2, A–H). Compared with controls, spleen gene expression of IL-1 $\alpha$  continued to significantly increase from  $T_{c,\text{Max}}$  through hypothermia (3.4- and 9.2-fold increase, respectively; ANOVA,  $P < 0.001$ ) and returned to baseline levels by 24 h (Fig. 2A). Liver IL-1 $\alpha$  gene expression levels showed a significant increase at hypothermia (4.3-fold increase) compared with controls and heated animals at  $T_{c,\text{Max}}$  and 24 h (ANOVA,  $P < 0.001$ ; Fig. 2B). By 24 h, IL-1 $\alpha$  returned to baseline levels in the liver (Fig. 2B).

Spleen IL-1 $\beta$  gene expression peaked at  $T_{c,\text{Max}}$  (4.4-fold increase) and continued to be expressed at significantly higher levels than controls throughout hypothermia (3.5-fold higher; ANOVA,  $P < 0.001$ ; Fig. 2C) but returned to baseline levels by 24 h. In comparison, liver IL-1 $\beta$  gene expression showed a progressive increase above control levels from  $T_{c,\text{Max}}$  to hypothermia (1.4- and 3.9-fold, respectively; ANOVA,  $P < 0.001$ ), before returning to control levels by 24 h (Fig. 2D).

IL-18 gene expression in the spleen showed a 2.5-fold increase above controls at  $T_{c,\text{Max}}$  and remained significantly elevated at hypothermia (1.7-fold increase; ANOVA,  $P < 0.001$ ; Fig. 2E). Spleen IL-18 levels were not significantly

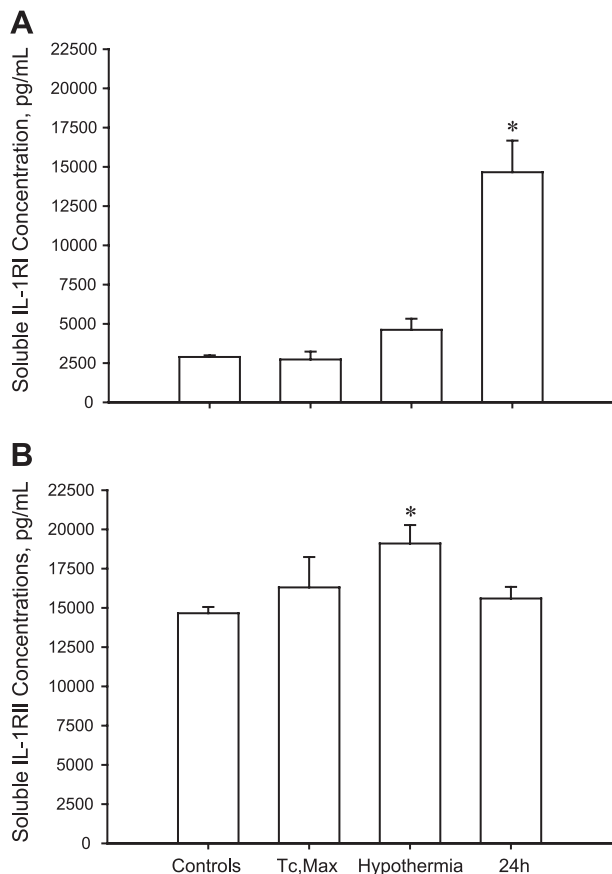


Fig. 1. Soluble IL-1 receptor expression in plasma samples from C57BL/6J mice. Protein expression of sIL-1RI (A) and sIL-1RII (B) measured by Multiplex analysis in controls, at maximum core temperature ( $T_{c,Max}$ ,  $n = 7$ ), hypothermia ( $n = 7$ ), and 24 h ( $n = 6$ ). Control values at each time point were similar among groups and combined for presentation purposes. \* $P < 0.05$  vs. all time points.

elevated above control levels at 24 h (Fig. 2E). In contrast to the majority of genes studied, expression of the IL-18 gene in the liver was significantly decreased at  $T_{c,Max}$  (ANOVA,  $P = 0.005$ ), hypothermia (ANOVA,  $P < 0.001$ ) and 24 h (ANOVA,  $P < 0.001$ ) compared with controls (Fig. 2F). Thus, liver IL-18 expression was down regulated in response to HS and did not return to baseline levels within the 24 h window of recovery.

Spleen IL-1RI gene expression was significantly higher at  $T_{c,Max}$  (2.4-fold increase; ANOVA,  $P < 0.001$ ) and remained elevated (3.5-fold increase; ANOVA,  $P < 0.001$ ) at hypothermia compared with controls (Fig. 2G). IL-1RI returned to baseline levels by 24 h (Fig. 2G). Within the liver, IL-1RI displayed significantly increased gene expression at  $T_{c,Max}$  (3.7-fold; ANOVA,  $P < 0.001$ ), hypothermia (5.4-fold; ANOVA,  $P < 0.001$ ) and 24 h (2.2-fold; ANOVA,  $P = 0.015$ ) compared with controls (Fig. 2H), but the levels at these three time points did not differ from one another. IL-1RII and IL-1RAcP expression was at minimum detection limits in spleen and liver samples at all time points tested (data not shown).

*T<sub>c</sub> responses of C57BL/6J and IL-1RI KO mice.* Figure 3 shows the  $T_c$  of C57BL/6J and IL-1RI KO mice during heat stress to a  $T_{c,Max}$  of 42.7°C and during ~48 h of recovery, a

time point that allowed for return of  $T_c$  values to baseline (preheat) in both strains. Control mice of both genotypes showed the normal circadian  $T_c$  profile with low daytime (~36°C) and elevated nighttime (~37°C) values (solid black lines). Note that the body-weighing procedure at *time 0* induced a transient ~1°C hyperthermia in control mice, but  $T_c$  returned to baseline levels within ~90 min. Weighing induced a similar hyperthermia in heat-stressed mice, followed by a steep increase in  $T_c$  due to an uncompensable environmental heat load as the heat stress  $T_a$  of 39.5°C was higher than  $T_c$ . Once  $T_c$  exceeded 39.5°C, mice were able to effectively dissipate body heat to the environment and a plateau in the  $T_c$  rise was observed (starting ~45 min, Fig. 3). Note that considerable variability was observed in the duration of the plateau, as previously described for our heat stress protocol (Fig. 3, Ref. 28). All mice were required to remain in the heat stress environment until they reached  $T_{c,Max}$  of 42.7°C and showed a subsequent steep rise in  $T_c$ , as  $T_{c,Max}$  was approached. Upon removal from the heat, C57BL/6J and IL-1RI KO mice showed a rapid decrease in  $T_c$  resulting in development of a ~8–10°C hypothermia compared with their nonheated controls.

All survivors began to recover from hypothermia between 360 and 480 min after HS began. Individual  $T_c$  curves were graphed in Fig. 3 to show differences in  $T_c$  response between survivors and nonsurvivors (C57BL/6J,  $n = 3$ ; IL-1RI KO,  $n = 2$ ) during the recovery period. Nonsurvivors succumbed to the effects of HS during recovery (360–720 min following initiation of HS), but not during the heating episode (Fig. 3, A and B). Table 1 quantifies the  $T_c$  response for C57BL/6J and IL-1RI KO mice during heat exposure and recovery. Despite accruing a greater heating area (IL-1RI KO  $298.5 \pm 15.7$  vs.  $246.8 \pm 13.4^\circ\text{C}\cdot\text{min}$  C57BL/6J,  $P < 0.05$ ), IL-1RI KO mice showed an attenuated hypothermia  $T_c$  ( $28.5 \pm 0.2$  vs.  $27.2 \pm 0.5^\circ\text{C}$ ,  $P < 0.05$ ), reduced time to  $T_{c,Min}$  ( $208.5 \pm 16.6$  vs.  $315.8 \pm 58.1$  min,  $P < 0.05$ ), duration of hypothermia ( $675.8 \pm 82.1$  vs.  $1,283.4 \pm 390.5$  min,  $P < 0.05$ ), and rewarming time ( $476.6 \pm 54.7$  vs.  $782.2 \pm 166.4$ ,  $P < 0.05$ ) with a higher  $T_c$  ( $36.9 \pm 0.5$  vs.  $34.1^\circ\text{C}$ ,  $P < 0.05$ ) 24 h following HS compared with C57BL/6J mice (Table 1). Despite these differences, C57BL/6J and IL-1RI KO mice experienced a similar level of dehydration during heat stress (11.1 vs. 10.0%, respectively; ANOVA,  $P = 0.08$ ), which was significantly elevated above that incurred by the nonheated control groups.

## DISCUSSION

The initial goal of this study was to determine plasma, liver, and spleen IL-1 family expression patterns in C57BL/6J mice, in which HS-induced tissue damage had been previously determined (27). The major findings of this study are threefold. First, plasma levels of sIL-1RI and sIL-1RII were elevated at 24 h and hypothermia, respectively. Second, spleen and liver IL-1 family gene expression was altered throughout 24 h of recovery following HS. Third, loss of IL-1 signaling increased the time to  $T_{c,Max}$  while improving all thermal indices of recovery.

Studies to date investigating the relationship between cytokines and HS have focused largely on the relationship between circulating levels of IL-1 and/or IL-1ra at the time of collapse and during recovery and their relation to mortality rates (6, 8, 9, 21, 36). A critical, but often overlooked aspect of IL-1-

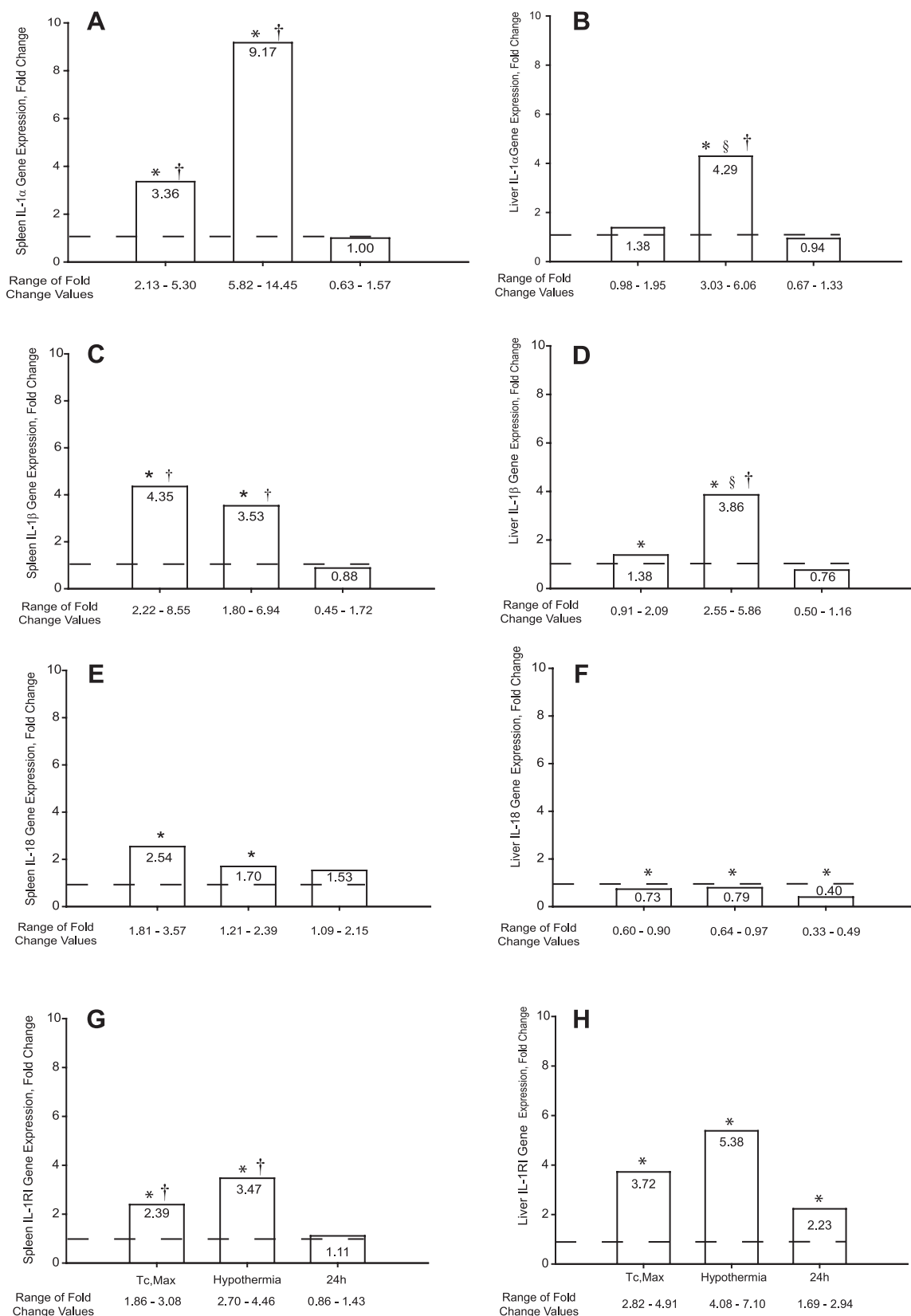


Fig. 2. Changes in spleen (left) and liver (right) gene expression of IL-1 $\alpha$  (A, B), IL-1 $\beta$  (C, D), IL-18 (E, F), IL-1RI (G, H) in C57BL/6J mice following HS. Gene expression was measured by real-time PCR in controls ( $n = 24$ ), at T<sub>c,Max</sub> ( $n = 7$ ), hypothermia ( $n = 7$ ), and 24 h ( $n = 6$ ). Values are mean fold change calculated from threshold cycle (Ct) values, with the range of fold change provided below each bar as described in MATERIALS AND METHODS. Control gene expression was similar among groups, assigned a value of 1, and combined for presentation purposes. \* $P < 0.05$  vs. controls; † $P < 0.05$  vs. 24 h; § $P < 0.05$  vs. T<sub>c,Max</sub>.

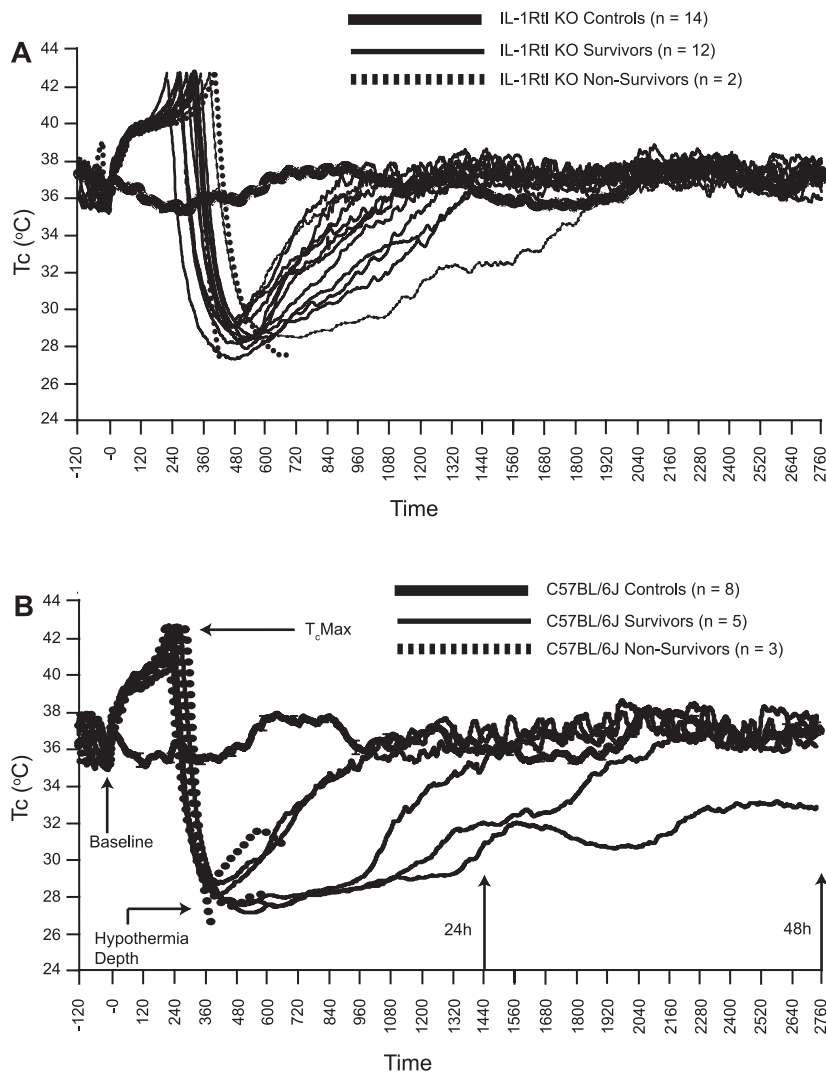


Fig. 3. Individual core temperature ( $T_c$ ) responses of IL-1RII knockout (KO) mice (A) and C57BL/6J mice (B) heat stressed at an ambient temperature ( $T_a$ ) = 39.5°C to a  $T_{c,Max}$  of 42.7 ± 0.0 °C compared with the  $T_c$  response of nonheat-stressed controls of the same genotype (solid black line on each graph). Housing and recovery were at  $T_a$  of 25 ± 2°C.

mediated physiological responses is the relationship of the endogenous cytokine to tissue and soluble forms of the receptor. Cleavage products of the transmembrane type I and II receptors result in the formation of sIL-1RI and sIL-1RII, which act as natural antagonists of IL-1 (2, 43). The presence of increased levels of circulating soluble receptors following HS expands previous studies using the same C57BL/6J mice population in which IL-1 $\beta$ , the high-affinity ligand for sIL-1RII, was elevated at hypothermia (27). Taken together, the results of current and previous studies suggest that elevations in

sIL-1RII at hypothermia may be an endogenous mechanism that works to enhance recovery from HS in wild-type (i.e., C57BL/6J) mice by sequestering excess circulating IL-1 $\beta$ . Interestingly, increased sIL-1RII levels are also found in septic patients (19), further supporting that HS is accompanied by a septic-like response in recovery. In comparison the sIL-1RI has the greatest affinity for the IL-1Ra followed by IL-1 $\alpha$  (13, 41). Although in the current study, at 24 h the sIL-1RI was significantly increased, circulating IL-1 $\alpha$  was not found to be elevated in the same animals (27). Unfortunately the limited

Table 1. Core temperature responses of heat-stressed IL-1RI KO and C57BL/6J mice

T <sub>c</sub> Response	IL-1RI KO Heat	Range	C57BL/6J Heat	Range
Time to T <sub>c,max</sub> , min	320.4 ± 14.0*	(222-404)	254.6 ± 8.5	(223-297)
Time to T <sub>c,min</sub> , min	208.5 ± 16.6*	(127-335)	315.8 ± 58.1	(164-653)
Hypothermia, °C	28.5 ± 0.2*	(28-30)	27.2 ± 0.5	(24-28)
Heating area, C·min	298.5 ± 15.7*	(194-392)	246.8 ± 13.4	(207-295)
Hypothermia duration, min	675.8 ± 82.1*	(333-1,307)	1283.4 ± 390.5	(565-2,657)
Rewarming time, min	476.6 ± 54.7*	(251-775)	782.2 ± 166.4	(433-801)
24 h T <sub>c</sub> , °C	36.9 ± 0.5*	(32-38)	34.1 ± 1.3	(29-37)

Core temperature ( $T_c$ ) responses were calculated for heating curves shown in Fig. 3 as described previously (20). Values are means ± SE with range of values shown in parentheses. \* $P$  < 0.05 vs. C57BL/6J mice. KO, knockout; T<sub>c,max</sub>, maximum core temperature; T<sub>c,min</sub>, lowest T<sub>c</sub> during recovery.



plasma volume in the mouse prevented measurements of IL-1Ra, and thus its relation to IL-1RI expression could not be determined in the current study.

The current study is unique in that it investigated the pathophysiology of HS at the tissue level following HS. Specifically, IL-1 family of cytokines and receptors was examined in the liver and spleen as the organs have significant roles in the innate immune response (16, 46) and are thought to have critical clearance roles with respect to the HS-induced endotoxin release (24). Furthermore, the organs show a different time course of injury with cytotoxic damage to the spleen present at  $T_{c,Max}$  (27). Consistent with the onset of damage, spleen IL-1 $\beta$  and IL-18 gene expression also peaked at  $T_{c,Max}$ , suggesting increased expression of these cytokines may be associated with HS morbidity in this organ. In contrast to spleen damage, the appearance of liver damage in our HS model was delayed (26), and the liver gene expression of IL-1 $\alpha$ , IL-1 $\beta$ , and IL1R1, along with IL-1 $\alpha$  and IL1R1 in the spleen, was maximal at hypothermia in the current study. Thus the elevated levels of IL-1 family gene expression at hypothermia may act internally to contribute to the delayed liver damage previously seen in C57BL/6J mice (26).

Although the organ-derived sources of circulating IL-1 and IL-1 receptors was not a focus of the current study, the increased gene expression in the liver and spleen suggests that the issues may contribute to circulating IL-1 family protein levels following HS. This may be particularly true of the spleen, an organ made up of red and white pulp with the latter comprising B and T lymphocytes and having a significant role in the immune response. However, in the absence of translational studies that include tagging proteins, the association of spleen and liver IL-1 and circulating levels is unclear. Instead the purpose of identifying IL-1 family gene expression was to determine if spleen and liver damage was associated with altered levels of IL-1 gene expression, an association that has been implicated in HS morbidity and mortality, albeit never investigated.

Surprisingly, we detected only very low levels of IL-1RII and were unable to detect IL-1RAcP gene expression in the spleen and liver. This finding may be related to a lack of expression in these tissues, the timing of transcription and translation, or methodological limitations. However, the latter seems unlikely as multiple primer sets were utilized in an effort to detect the accessory portion of the receptor complex.

Gene expression of IL-18, a recently discovered IL-1 family member that possesses potent proinflammatory properties, was examined for the first time in the context of HS and was shown to be increased in the spleen at  $T_{c,Max}$  and hypothermia but decreased in the liver at all time points. IL-18 interacts with IL-12 to induce cell-mediated immunity in response to endotoxin (e.g., LPS; Refs. 3, 14). Previously we observed elevated plasma IL-12 levels at  $T_{c,Max}$ , and it is intriguing to speculate that IL-18/IL-12 interactions are occurring in our HS model (27). However, we were unable to examine plasma IL-18 levels in the current study due to a lack of mouse reagents for this cytokine, and we did not measure IL-12 in our spleen samples as that was beyond the scope of this study.

In a comparison of the  $\alpha$ - and  $\beta$ -isoforms of IL-1 gene expression, the contributions and role of IL-1 $\alpha$  are not well known, although release of the cytokine has been shown to occur in response to endotoxin injection (4) and severe inflam-

mation (1, 12). With regard to heat, in animals exposed to a hyperthermic environment followed by endotoxin challenge 6 h later, IL-1 $\alpha$  was threefold greater than in animals not previously exposed to heat. Furthermore, upon admission for HS and following cooling, circulating levels of IL-1 $\alpha$  are elevated (8) and thought to act in synergy with TNF- $\alpha$  to mediate pathophysiology associated with endotoxin exposure (8). Interestingly pretreatment with IL-1 $\alpha$  was shown to offer end-organ protection when administered to female mice prior to an endotoxin challenge meant to mimic sepsis (25). The reduced damage was associated with alterations in IL-6 and TNF- $\alpha$ , demonstrating the importance of the cytokine milieu (25). Thus, future studies determining how the absence of IL-1 signaling disrupts IL-1/TNF- $\alpha$ /IL-6 interactions will be of value in determining the role of cytokines following HS.

A number of studies have examined IL-1 $\beta$  in the context of HS but offer conflicting results regarding the expression and role of the cytokine. For instance, in an anesthetized baboon model of HS, Bouchama et al. (7) were unable to detect IL-1 $\beta$  at baseline or up to 3 h following moderate (100% survival) or severe (0% survival) HS. However, work by the same group in humans has shown IL-1 $\beta$  elevations in 39% of HS cases (6), while our conscious mouse model revealed elevated IL-1 $\beta$  levels following HS (27). Lin and colleagues, in a series of papers, have shown that HS is accompanied by elevations in circulating IL-1 $\beta$  levels in anesthetized rats (10, 11, 33, 34) and anesthetized rabbits (32). Differences in the timing of sample collection, use of anesthesia, species selection, and administration of the IL-1ra prevent formation of a clear conclusion regarding the relationship between IL-1 and HS. The current study sought to expand previous findings by determining the influence of IL-1 during HS onset and recovery. Multiple factors were controlled for in the current study including complete removal of IL-1-mediated actions via a KO model, the use of a conscious preparation, which allows for maintenance of normal thermoregulatory mechanisms (22, 42), and collection of blood and tissues at multiple time points.

Several findings from the current results implicate IL-1 cytokines as having a critical role in organ damage related to delayed recovery following HS. First, IL-1RI KO mice experienced a greater heating area, yet showed a tendency toward a faster cooling rate (0.07 vs. 0.05°C/min, respectively). Thus IL-1RI KO animals, lacking the ability to signal via IL-1 cytokines, were more effective at heat dissipation compared with C57BL/6J animals and appeared to be more tolerant to HS than C57BL/6J mice. Second, compared with C57BL/6J mice, IL-1RI KO mice showed a reduction in the duration of their hypothermia bout and rewarmed significantly faster from hypothermia to  $T_c$  of 34.5°C, the lowest  $T_c$  value observed in an undisturbed nonheated mouse before heat stress (28). Results of our previous work have shown that the duration of hypothermia and rewarming time has a direct relationship to organ damage (27). Compared with C57BL/6J mice, IL-1RI KO animals displayed decreased hypothermia, decreased hypothermia duration, and faster rewarming to baseline  $T_c$ , all indexes of improved thermal tolerance in the absence of endogenous IL-1 actions. Third, IL-1RI KO mice displayed fever at 24 h of recovery, while many C57BL/6J mice remained hypothermic, a response that serves as a marker of morbidity (28). By 48 h, IL-1RI KO  $T_c$  had returned to baseline, whereas C57BL/6J mice showed a tendency toward fever compared with their



nonheated controls. Compared with IL-1RI KO mice, the delayed and variable transition from hypothermia toward a fever-like state in C57BL/6J mice is consistent with our previous findings in this strain (28). Determination of the physiological mechanisms contributing to the leftward shift in the recovery profile of IL-1RI KO mice was not a goal of this study. However, IL-1 $\beta$  is known to contribute to fever development following LPS injection (5) and has been shown to induce fever when injected into the intracerebroventricular space (40). Furthermore, systemic administration of LPS increases IL-1 $\beta$  mRNA expression in the rat anterior hypothalamus (38), while thermosensitive neurons in the same region are influenced by the cytokine (47). Specifically, the presence of IL-1 $\beta$  reduces the sensitivity of temperature-sensitive neurons in the anterior hypothalamus and decreases their firing frequency (45), an effect that is attenuated when the IL-1ra is given (47). Collectively these studies combined with our earlier work showing that following HS mice experience fever (29) suggest that IL-1 may modulate thermoregulatory effector responses during heat exposure and provide an immune (i.e., IL-1) link to central responses associated with HS.

A caveat in the current study regarding experimental design should be noted. The  $T_c$  patterns of HS mice were followed for 48 h to compare differences in HS recovery between knockout (IL-1RI KO) and wild-type (C57BL/6J) mice. In contrast the C57BL/6J mouse tissues for which gene expression data are presented were harvested as part of a previous protocol in which animals were tracked for 24 h post-HS (27). Studying recovery in IL-1RI KO compared with C57BL/6J animals was a goal of this study, hence we tracked animals over a 48 h time period, a time that allowed recovery of  $T_c$  to baseline values in both strains. Contrasting this, instead of determining gene expression patterns in IL-1RI KO mice, we sought to track gene expression changes at predefined  $T_c$  biomarkers in those animals (i.e., C57BL/6J) in which tissue damage had already been determined (26, 27).

While the current study provides evidence suggesting the IL-1 family of cytokines plays a significant role in the morbidity and recovery associated with HS, future studies that investigate changes in the cytokine milieu are needed to better understand additive, synergistic, or competing actions of these proteins in understanding the pathophysiology and SIRS-like responses associated with HS. In addition, future studies designed to measure tissue protein levels would provide more direct evidence in support of a role for IL-1 in support of morbidity associated with HS. It would also be beneficial to examine other organs known to be involved in the SIRS (e.g., brain, kidney).

### Perspectives

Collectively, these studies provide insight into a molecular mechanism that may serve as a future therapeutic target for HS patients. Although the role of cytokines in HS recovery is not well understood, the use of knockout models provides unique insight into the pathophysiology of HS and the role cytokines play during recovery. Furthermore, the use of knockout animals avoids the pharmacological challenges encountered when using antibodies and peptides to block the actions of cytokines allowing for a more in-depth study of the actions of a single cytokine. However, because cytokines have redundant action

and rarely act in isolation, studies in wild-type animals will continue to be critical in understanding the contributions of the cytokine milieu to HS recovery.

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### DISCLOSURES

No conflicts of interest (financial or otherwise) are declared by the author(s).

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