Acute immune responses are involved in liver and kidney injury in heat stroke

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Abstract
The changes in circulating cytokines throughout the progression of heat stroke–induced systemic inflammatory response syndrome have been thoroughly described. However, the innate and adaptive immune responses were poorly understood and are explored in this study. A classic heat stroke model in mice was constructed according to the established methods. The time course of splenic T helper cell plasticity, neutrophils, and macrophages in liver and kidney tissues were measured at 0, 24, and 72 h after onset of heat stroke. We showed heat stroke mice presented tachycardia and low mean arterial pressure and exhibited severe kidney and liver injury. Our data demonstrated that heat stroke could increase both the Th17 and Th22 response of splenic T helper cells and incremental infiltration of neutrophils and macrophages into liver and kidney tissues. Moreover, heat stroke could shift those macrophages into the M1 type. This study demonstrates for the first time the increasing splenic T helper response, changes in neutrophil and macrophage infiltration, and macrophage induction in response to heat stroke in mice. Our findings indicate that splenic T helper cells and local neutrophils and macrophages might be potential therapeutic targets for heat stroke.

Keywords
cytokines, heat stroke, macrophages, neutrophils, T helper cells

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Introduction
Heat stroke (HS) is a life-threatening illness that is characterized clinically by central nervous system dysfunction, hyperthermia, and multiple organ dysfunction.1,2 Despite rapid cooling and functional multi-organ support, many patients still experience permanent neurological impairments or death.3 HS presents overwhelming systemic inflammatory response syndrome (SIRS), but there is currently limited understanding of the endogenous mechanisms. However, few studies have focused on the innate and adaptive immune responses to HS in the spleen, liver, and kidneys.

T helper cells (Th cells) are a classical but constantly reinterpreted T-cell subset, playing critical roles in a diverse range of inflammatory or disease responses. Many previous studies proved that Th cells play important roles in sepsis or HS;4,5 however, none of those studies detected cell plasticity in Th cells during HS, such as Th1/Th2/Th17/Th22/Treg cells. Macrophages hold a critical position in the pathogenesis of liver and kidney injury and repair. Based on distinct phenotypes and origins, hepatic macrophages are capable of clearing...
pathogens and of promoting or inhibiting liver inflammation via releasing different types of pro- or anti-inflammatory cytokines and growth factors. Until now, no study has carefully examined the role of macrophages and their polarization in local organs during HS, though Lim and Mackinnon found an increase of macrophage activity due to muscle tissue damage in exertional HS. Neutrophils also play a pivotal role in tissue injury. Geng et al. demonstrated that interleukin (IL)-1β could trigger neutrophil tissue infiltration and lead to liver damage. Although human and animal studies demonstrated botryoid neutrophils and characteristic of HS, in lung tissues or peripheral blood smears, few studies have examined local neutrophil response in liver and kidney tissues during HS.

The aim of this study was to examine the innate and adaptive immune responses to HS, including splenic Th cell plasticity and neutrophil and macrophage response to HS in the liver and kidneys, to provide novel therapeutic strategies.

**Materials and methods**

**Animals and procedures**

C57/BL6 male mice (20–25 g; 8–12 weeks) were purchased from the animal center at the Chinese PLA General Hospital. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Chinese PLA General Hospital.

Mice were divided into HS and control groups. Heat stress protocol was administered as described in previous studies. Briefly, mice were exposed to an incubator temperature (Ta) of 42°C ± 0.2°C with humidity of 50% ± 5% in the absence of food and water, and rectal temperature was monitored every 10 min using a digital thermometer (ALC-ET06, Shanghai Alcott Biotech Co, Shanghai, China) inserted 2 cm into the rectum until a maximum temperature of 42.7°C was attained, the criterion for moderate HS. Mice were then removed from the chamber and allowed to passively recover at room temperature (25°C ± 0.5°C) in a new cage with food and water given ad libitum. The control mice underwent the same experimental procedure as the HS mice but with the chamber temperature maintained at 25°C ± 0.5°C and relative humidity at 50% ± 5% throughout the experiment.

At 0, 24, or 72 h after HS, the mice were euthanized with an intraperitoneal injection of phenobarbital. Mean arterial blood pressure (MAP) and heart rate (HR) were detected via non-invasive blood pressure monitoring through the tail artery. Blood, spleen, kidney, and liver samples were harvested immediately following blood withdrawal for further processing.

**Histopathological examination**

Kidney and liver samples were sliced into transverse or longitudinal sections and fixed in 10% neutral-buffered formalin. The tissues were then embedded in paraffin blocks, and serial sections were stained with hematoxylin and eosin for microscopic evaluation at a magnification of ×200.

**Cytokine assay**

Cytokines secreted by splenic cells determining Th cell subsets, such as interferon (IFN)-γ, IL-4, IL-10, IL-17, IL-22, and IL-6, were detected using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 17-Plex Mouse ProcartaPlex Panel according to the manufacturer’s instructions.

**Cell preparation**

Single-cell preparations from spleen, liver, and kidney were performed according to established methods. Blood was flushed out with ice-cold phosphate-buffered saline (PBS); and spleens, livers, and kidneys were harvested. Spleen and liver tissue was removed, minced in PBS with 1% bovine serum albumin (BSA) and lysed with red blood cell (RBC) lysis buffer (TBD Science, Tianjing, China) for 1–2 min, according to the manufacturer’s protocols. The kidney was decapsulated, diced, and incubated at 37°C for 30 min with collagenase I (0.5 mg/mL; Sigma-Aldrich, China) and DNaseI (100 U/mL; Sigma-Aldrich) in Hank’s balanced salt solution (HBSS). To remove debris, samples were filtered through mesh (40 mm). Single cells were washed in PBS for supplementary application.

**Flow cytometric analysis**

We performed surface staining with the antibodies FITC-CD4 (553729, BD, Brentwood, TN, USA), PE anti-mouse F4/80 (Biolegend, BM8, San Diego, CA, USA), PE anti-mouse/human CD11b (Biolegend, M1/70), and FITC anti-mouse Ly-6 G (Biolegend, 1A8) according to the manufacturer’s instructions.
instructions. We performed intracellular staining for Foxp3 with APC-Foxp3 (17-5773-80, eBioscience, San Diego, CA, USA) according to the manufacturer’s instructions. We performed intracellular staining for iNOS and arginase 1 with the antibodies anti-Mouse NOS2 APC (eBioscience, CXNFT) and Human/Mouse Arginase 1/ARG1 fluorescein conjugated (R&D Systems, Polyclonal, Minneapolis, MN, USA) after stimulation with a cell stimulation cocktail plus protein transport inhibitors (eBioscience) for 16 h, according to the manufacturer’s protocol. IFN-γ, IL-4, IL-17, and IL-22 were detected by intracellular staining with the following antibodies: anti-mouse IFN-γPE (eBioscience, XMG1.2), anti-mouse IL-4 PE (eBioscience, 11B11), anti-mouse IL-17A PE (eBioscience, eBio17B7), and anti-mouse IL-22 PE (eBioscience, 1H8PWSR) according to the manufacturer’s protocols. Cells were observed using BD FACSCalibur (BD Immunocytometry Systems, Brentwood, TN, USA) and analyzed with FlowJo 7.6 software.

**Immunofluorescence staining**

For immunofluorescence, sections were fixed in acetone at −20°C for 10 min, followed by blocking with 1× casein for 30 min at room temperature. The primary antibodies Rat anti-mice Ly-6G (1:100) (eBioscience) and Rat anti-mice F4/80 (1:100) (eBioscience) were incubated overnight at 4°C. After washing in PBS three times, the sections were incubated with FITC/PE-conjugated AffiniPure donkey anti-rat IgG (1/50; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Rat IgG was used as an isotype-matched control. Sections were mounted with mounting medium containing DAPI (4’,6-diamidino-2-phenylindole; Zhongshan Goldenbridge Biotechnology, Beijing, China). Each tissue section was observed under a confocal laser-scanning microscope (Olympus FluoView 1000, Tokyo, Japan) at magnifications of 600 and 2400×, if necessary.

**Statistical analysis**

Analysis was performed using the IBM SPSS Statistics 18.0 software (IBM Corporation, Armonk, NY, USA). Results are presented as mean values ± standard deviation (SD). Multiple comparisons of parametric data were performed using one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls (SNK) post hoc tests. Student’s t-test was used to compare differences in means. Non-parametric data were compared with the Mann–Whitney U-test to identify differences between groups, and α was corrected by the number of comparisons (α/comparisons) to ensure α=0.05. *P<0.05, ** 0.001<P<0.01, ***P<0.001. Graphs were made via GraphPad Prism5 software.

**Results**

**HS symptoms**

Patients with HS show symptoms of coma, hyperthermia, tachycardia, low systolic blood pressure, and subsequent multiple organ dysfunction, including acute liver and kidney injury, coagulopathy, and cardiac depression. In our rodent HS model, mice in the HS group had core temperatures reaching 42.7°C in addition to lower mean arterial pressure (Figure 1(a) and (b)) and faster HR (Figure 1(c) and (d)) than the mice in the control group at every time point. HS was characterized as progressive multiple organ dysfunction, including acute injury to the kidneys and liver. Serum biomarker levels of liver (alanine transaminase (ALT) and aspartate transaminase (AST), Figure 1(e)–(g)) and kidney function (SCr and BUN, Figure 1(i)–(k)) were significantly higher for the HS group than for the control group at 24 and 72 h. Hepatocytes from HS mice showed visible necrosis, ballooning degeneration, and liver hemorrhage (Figure 1(g)), and typical renal histopathology injuries were observed primarily in the cortex, characterized by visible vascular congestion, hemorrhage, and thrombi (Figure 1(l)).

**HS could promote splenic Th cell differentiation toward Th17 and Th22 subsets and decrease splenic Treg frequency**

Intracellular staining via fluorescence-activated cell sorting (FACS) showed that the percentage of CD4⁺ IFNγ (Figure 2(a) and (b)) peaked at 0 h, was still elevated at 24 h, and then decreased for both groups. Mice in the HS group had a higher percentage of CD4⁺ IL-4⁺ (Figure 2(c) and (d)) across time. Furthermore, the percentage of CD4⁺ IL-17⁺ (Figure 2(e) and (f)) and CD4⁺ IL-22⁺ (Figure 2(g) and (h)) cells were significantly higher across the study period for the HS group.
Figure 1. Mice in the heat stroke group presented lower mean arterial pressure, higher heart rate, and acute liver and kidney injury: (a, b) time course of mean arterial pressure (n = 10); (c, d) time course of heart rate (n = 10); (e, f, h) time course of ALT and AST (n = 6); (g): HE staining of liver from HS mice showed visible hepatocyte necrosis, ballooning degeneration, and liver hemorrhage (n = 6, ×200); (i, j, k) time course of SCr and BUN (n = 6); and (l) HE staining of kidney from HS mice showed vascular congestion, hemorrhage, and thrombi, primarily in cortex (n = 6, ×200).

N.S.: not significant; MAP: mean arterial pressure; HR: heart rate; ALT: alanine aminotransferase; AST: aspartate aminotransferase; SCr: serum creatinine; BUN: blood urea nitrogen; NC: negative control; HS: heat stroke.

Data are expressed as mean ± SD; P values are adjusted for multiple comparisons.

*P < 0.05; **P < 0.01; ***P < 0.001.
Figure 2. Heat stroke could promote splenic Th cell differentiation toward Th17 and Th22 subsets and decrease splenic Treg frequency: (a, b) intracellular staining of IFNγ detected by FACS; (c, d) intracellular staining of IL-4 detected by FACS; (e, f) intracellular staining of IL-17 detected by FACS; (g, h) intracellular staining of IL-22 detected by FACS; (i, j) splenic Tregs frequency detected by FACS; and (k–q) Th cell–related cytokines in culture supernatants of splenocytes detected with Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit.

N.S.: not significant; IFNγ: interferon-γ; IL-4: interleukin-4; IL-17: interleukin-17; IL-22: interleukin-22; NC: negative control; HS: heat stroke (n = 5); IL-22: interleukin-22; IL-6: interleukin-6; IL-10: interleukin-10; NC: negative control. Data are expressed as mean ± SD; P values are adjusted for multiple comparisons. "P < 0.05; "**P < 0.001.

Regulatory T cells (CD4+ Foxp3+) decreased over time for the HS group (Figure 2(i) and (j)). IFNγ, a metric of splenic Th cell plasticity, increased immediately, peaked at 0 h, and decreased with time in supernatants of cultured splenic cells from mice in the HS group. At 72 h, no marked increase of IFNγ was observed in our study (Figure 2(k)). IL-4 (Figure 2(l)) and IL-22 (Figure 2(o)) were increased at 24 and 72 h in the HS group, peaking at 24 h. IL-17 (Figure 2(m)) and IL-10 (Figure 2(q)) showed significant increases at 0, 24, and 72 h for the HS group, peaking at 24 h. IL-6 increased at every time point for the HS group and peaked at 72 h.

**HS increases the percentage of macrophages in liver and kidney, shifts those macrophages into the M1 type and increases proportion of neutrophils in liver and kidney**

The percentage of macrophages (F4/80+) increased significantly in liver tissue (Figure 3(a) and (b)) at every time point and peaked at 24 h for the HS group, while the percentage of macrophages (F4/80+) increased significantly in the kidneys (Figure 3(c) and (d)) at 24 and 72 h.

Macrophage polarization, vital in pathogenesis of sepsis, of M1 macrophages (F4/80+ iNOS+, data not shown) increased at 24 and 72 h after HS onset, and peaked at 72 h. The percentage of M2 macrophages (F4/80+ Arginase+, data not shown) decreased over time in the liver. Furthermore, the M2/M1 ratio decreased at 24 and 72 h, compared to that of the control group (Figure 3(e)). The percentage of M1 macrophages (F4/80+ iNOS+, data not shown) increased at every time point and peaked at 72 h in the kidney, while the percentage of M2 macrophages (F4/80+ Arginase+, data not shown) decreased over time in the kidney. Moreover, the M2/M1 ratio decreased across every time point, compared to the control group (Figure 3(f)).

Neutrophils (CD11b+ Ly-6G+), involved in liver and kidney injury in HS, increased significantly in liver (Figure 3(g) and (h)) and kidney (Figure 3(i) and (j)) at 24 and 72 h after heat stress and peaked at 24 h.

**Discussion**

Our study explored for the first time innate and adaptive immune responses to HS, including splenic Th cell plasticity, and neutrophil and macrophage response to HS in liver and kidney tissues, which might be involved in multiple organ injury and thus become potential therapeutic targets for HS. Emerging evidence suggests that the multi-organ damage resulting from HS is a consequence of SIRS stimulated by endotoxin, cytokines, and other immune modulators. High circulating levels of cytokines and chemokines correlate with HS morbidity and mortality, and plasma interferon (IFN)-γ, interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)α, IL-8, and monocyte chemo-attractant protein (MCP)-1 plays important roles in multi-organ dysfunction in HS. Our study showed similar patterns for most of these cytokines and chemokines (data not shown). Despite these efforts, there remain knowledge gaps in the understanding of the mechanisms mediating multi-organ damage. Thus, novel pathogenesis, such as that of the innate and adaptive immune system, should be explored to improve treatment of this life-threatening disease.

Th cells dominate in SIRS-related diseases, such as sepsis. However, little was known about how Th cell plasticity orchestrated SIRS in HS. In our study, cell intracellular staining showed that the percentage of CD4+ IL-17+ cells increased after heat stress indicating augmentation of the Th17 response. Strikingly increased plasma transforming growth factor (TGF)-β, IL-6, and IL-1β (data not shown) might explain the boosting of splenic Th cells shift to Th17 response. Similarly, Th22 response was augmented after HS. Increased...
Figure 3. (Continued)
Figure 3. Heat stroke induces macrophage and neutrophil infiltration increased in liver and kidney and a shift of macrophages to M1 phenotype: (a, b) macrophages (F4/80+) infiltrated in liver detected by FACS. Cells were stained with PE anti-mouse F4/80 Antibody; (c, d) macrophages (F4/80+) infiltrated in kidney detected by FACS; (e) M2/M1 ratio in liver; (f) M2/M1 ratio in kidney; and (g, h) neutrophils (CD11b+ Ly-6G+) infiltrated in liver detected by FACs. Cells were stained with PE anti-mouse/human CD11b and FITC anti-mouse Ly-6G antibodies. (i, j) neutrophils (CD11b+ Ly-6G+) infiltrated in kidney detected by FACs. N.S.: not significant; NC: negative control; HS: heat stroke (n=5).

Data are expressed as mean ± SD; P values are adjusted for multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001.

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