Exposure to febrile temperature upregulates expression of pyrogenic cytokines in endotoxin-challenged mice

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Jiang, Qingqi, Louis Detolla, Ishwar S. Singh, Lisa Gatdula, Bridget Fitzgerald, Nico Van Rooijen, Alan S. Cross, and Jeffrey D. Hasday. Exposure to febrile temperature upregulates expression of pyrogenic cytokines in endotoxin-challenged mice. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1653–R1660, 1999.—Fever is a phylogenetically ancient response that is associated with improved survival in acute infections. In endothermic animals, fever is induced by a set of pyrogenic cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6] that are also essential for survival in acute infections. We studied the influence of core temperature on cytokine expression using an anesthetized mouse model in which core temperature was adjusted by immersion in water baths. We showed that raising core temperature from basal (36.5–37.5°C) to febrile (39.5–40°C) levels increased peak plasma TNF-α and IL-6 levels by 4.1- and 2.7-fold, respectively, and changed the kinetics of IL-1β expression in response to lipopolysaccharide challenge. TNF-α levels were increased predominantly in liver, IL-1β levels were higher in lung, and IL-6 levels were widely increased in multiple organs in the warmer mice. This demonstrates that the thermal component of fever may directly contribute to shaping the host response by regulating the timing, magnitude, and tissue distribution of cytokine generation during the acute-phase response.

Fever: body temperature; tumor necrosis factor-α; interleukin-1; interleukin-6; Kupffer cells; heat shock proteins

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endotoxin [lipopolysaccharide (LPS)]. The objective of this study was to determine how shifting core temperature from basal to febrile levels modifies the early cytokine response to LPS. Using an anesthetized, temperature-clamped mouse model, we found that increasing core temperature from 37 to 39.5–40°C caused an early, amplified, but self-limited pulse of TNF-α expression and persistent enhancement of IL-6 production in most tissues while preventing coexpression of TNF-α and IL-1β expression. In contrast with TNF-α expression, which was largely confined to the hepatic Kupffer cells, IL-6 expression was widely enhanced in the warmer animals. Finally, we showed that the temperature-dependent changes in cytokine expression were associated with only a partial activation of heat shock protein (HSP)-72 expression.

METHODS

Reagents

LPS prepared by trichloroacetic acid extraction from Escherichia coli 0111:B4 was purchased from DIFCO Laboratories (Detroit, MI). Avertin (2,2,2-tribromoethyl) was obtained from Sigma Chemical (St. Louis, MO).

Temperature Clamping

Six- to eight-week-old male CD-1 mice, weighing 25–30 g, were purchased from Harlan Sprague Dawley (Indianapolis, IN), housed in the University of Maryland, Baltimore, animal facility under the supervision of a full-time veterinarian, and used within 4 wk. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. Mice were anesthetized with 30 mg/kg tribromoethanol (Sigma) administered subcutaneously. The anesthetized animals were suspended in water baths (VWR; temperature variation <0.2°C) to the level of the axillae. Body temperature was continuously monitored with rectal thermistors. When body temperature reached bath temperature, 0.25 ml of a dose of LPS (see RESULTS for dose used in each experiment) or vehicle (pyrogen-free saline) was administered as an intraperitoneal injection. To control for the effects of anesthesia and water immersion, we also studied a group of conscious, unrestrained mice at normal laboratory temperatures (22–24°C). To model endotoxemia or bacteremia in the setting of established infection in febrile hosts, we increased core temperature to febrile levels before administering LPS. Core temperature reached water bath temperature in <10 min and varied by <0.2°C during the experiments. To avoid the influence of diurnal cycling, all experiments were started at approximately the same time each day (between 8:00 AM and 10:00 AM).

Plasma Cytokine Levels

Mouse TNF-α, IL-1β, and IL-6 were measured in the University of Maryland, Baltimore, Cytokine Core Laboratory using standard two-antibody ELISA with commercial antibody pairs and recombinant standards (TNF-α and IL-6 from Endogen, Boston, MA; and IL-1β from Genzyme, Cambridge, MA). Poly styrene plates (Maxisorb; Nunc) were coated with capture antibody in PBS overnight at 25°C. The plates were washed four times with 50 mM Tris, 0.2% Tween 20, pH 7.2 and then blocked for 90 min at 25°C with assay buffer (PBS containing 4% BSA and 0.01% thimerosal, pH 7.2). The plates were washed, and 50 µl of assay buffer were added to each along with 50 µl of sample or standard prepared in assay buffer and incubated at 37°C for 2 h. After the plates were washed, strepavidin-peroxidase polymer in casein buffer (Research Diagnostics, Mount Pleasant, NJ) was added and incubated at 25°C for 30 min. The plate was washed, and 100 µl substrate (TMB; Dako, Carpenteria, CA) was added and incubated for 20–30 min. The reaction was stopped with 100 µl 2N HCl, and the absorbance at 450 nm (minus absorbance at 650 nm) was read on a microplate reader ( Molecular Devices, Sunnyvale, CA). The data were analyzed using a computer program (SoftPro; Molecular Devices). These assays had lower detection limits of 8, 3, and 1.5 pg/ml, respectively.

Organ-Specific Cytokine Analysis

Spleen, lungs, liver, and kidney were collected and snap-frozen after flushing the circulation with 10 ml of 4°C PBS containing protease inhibitors (4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml leupeptin) injected through the left ventricle and drained from the left atrium. Organs were powdered under liquid nitrogen, homogenized in 1 ml lysing buffer (150 mM NaCl, 25 mM Tris, pH 7.4, 1% Nonidet P-40, 4 mM EDTA, and protease inhibitors), and cleared by centrifugation. Total protein concentration in the homogenate supernatants was measured using a commercial reagent (Bio-Rad, Mountainview, CA) with BSA (Sigma) as a standard. To minimize the background signal from endogenous biotin-containing proteins, 50 µl of a 3 mg/ml avidin solution (Sigma) in PBS was added to 470 µl of each homogenate supernatant. The treated homogenates were analyzed by ELISA as described above except for an additional 20-min incubation with 20 µg/ml free biotin (Sigma) in PBS added just before the detecting antibody. This method reduced background noise by >99% without affecting specific cytokine signal. Cytokine concentrations in homogenate supernatants were standardized to total protein concentrations.

Immunoblot Analysis of HSP-72

Homogenates were prepared from liver and kidney exactly as described for the organ ELISA analysis. Homogenates containing 200 µg total protein were separated on 12% Laemmli SDS-PAGE under reducing conditions, electrostatically transferred to polyvinylidene difluoride membrane (Stratagene), blocked with 5% dry milk in PBS-0.01% (vol/vol) Tween 20 overnight at 4°C, and incubated with 1:16,000 dilution of anti-HSP-72 (product no. SPA-810; StressGen, Victoria, BC, Canada) in blocking buffer for 2 h at room temperature. Bands were detected using 1:2,000 dilution of goat anti-mouse IgG peroxidase conjugate (Transduction Laboratories, Lexington, KY) in blocking buffer for 1 h at room temperature and a chemiluminescence detection system (New England Nuclear, Boston, MA). Intensities of autoradiograph bands were quantified using an Ambis (Billerica, MA) biological image analysis system. Sonicates from L929 cells incubated at 42°C for 90 min and then at 37°C for an additional 18 h were used as a positive control for HSP-72.

Experimental Protocols

Experiment 1: Effect of core temperature on plasma cytokine levels after LPS challenge. Three groups of six mice each were studied. One group of animals ("undamped") was injected intraperitoneally with 50 µg LPS and returned to the cages housed at normal ambient temperature (22–24°C). These mice maintained core temperatures of 35.5–36°C during the course of the experiment. A second group of mice was temperature clamped at 37°C and treated with 50 µg LPS. A third...
RESULTS

Effect of Core Temperature on LPS-Induced Cytokine Expression

The influence of core temperature on expression of early response cytokines was studied using the LPS-challenged, temperature-clamped mouse model. Peak plasma TNF-α levels were 4.1-fold higher and peaked 1 h earlier in the 40°C mice compared with the 37°C animals (Fig. 1A). Circulating TNF-α levels were similar in the unclamped and the 37°C clamped mice. As expected, circulating IL-6 appeared 1–2 h after TNF-α. Raising core temperature from 37 to 40°C increased peak plasma IL-6 levels 2.7-fold (Fig. 1D) but did not alter the kinetics of IL-6 expression. The effect of raising core temperature on circulating IL-1β expression was more complex. In 37°C clamped mice, circulating IL-1β expression was biphasic, with peaks occurring 1 and 4 h after LPS challenge (Fig. 2). Increasing core temperature to 40°C attenuated the early IL-1β peak and delayed the late IL-1β peak, thereby temporally separating TNF-α and IL-1β expression.

Plasma TNF-α, IL-6, and IL-1β were not detectable in control mice temperature clamped at either 37 or 40°C without LPS treatment, suggesting that core temperature changes could modify but not directly induce proinflammatory cytokine expression in the absence of pyrogen.

Influence of Core Temperature on LPS Dose Response for Plasma TNF-α and IL-6 Expression

The LPS dose response for both circulating TNF-α and IL-6 was flat between 50 and 250 µg in 37°C clamped mice (Fig. 1, B and E), but the dose response remained steeply positive over this dose range in the 40°C animals. In mice injected with 250 µg LPS, peak plasma TNF-α levels were 12.3-fold higher and IL-6 levels were 3.9-fold higher in the 40 vs. 37°C animals.

Effect of Intermediate Core Temperatures on Plasma IL-6 Expression

To define the threshold temperature required to amplify TNF-α and IL-6 expression, we compared plasma TNF-α and IL-6 levels 1 or 3 h after LPS challenge, respectively, in mice maintained between 37 and 40°C (Fig. 1, C and F). The threshold temperature for enhancing plasma levels of both TNF-α and IL-6 consistently occurred between 39 and 39.5°C, a temperature within the murine febrile range (18).

Effect of Transient Shifts in Core Temperature on Plasma TNF-α and IL-6 Expression

To simulate a more transient fever, mice were clamped at 37 or 40°C for only 1 h after receiving 50 µg LPS and were killed 5 h later. At death, plasma IL-6 levels were 6.5-fold higher in the animals transiently warmed to 40°C (220 ± 46 vs. 34.4 ± 5.3 ng/ml; P < 0.01). Plasma TNF-α was not detectable at this time point in either group.

Effect of Core Temperature on Levels of Organ-Associated Cytokines

We previously demonstrated that hepatic Kupffer cells were the predominant temperature-dependent source of LPS-induced TNF-α production. To compare the potential effects of fever on tissue distribution of
IL-1β, IL-6, and TNF-α, we measured concentrations of these cytokines in homogenates of liver, spleen, kidney, and lung obtained from the LPS-challenged, temperature-clamped animals (Fig. 3). These organs were selected for study because they are either important sources of systemic cytokine expression or are frequently injured in sepsis (8). As we previously found (21), the liver was the only tissue in which TNF-α levels were increased in 40 vs. 37°C mice after LPS challenge (Fig. 3A). By comparison, LPS-induced IL-6 levels were higher in spleen (2.6-fold) and lung (3.4-fold) as well as in liver (15-fold) in the 40°C mice compared with the 37°C animals (Fig. 3B). Raising core temperature from 37 to 40°C increased IL-1β levels in the lungs but not in the liver, spleen, or kidneys of LPS-challenged mice (Fig. 3C). Clamping core temperature at 40°C without LPS challenge caused a slow increase in hepatic TNF-α content but did not affect TNF-α levels in spleen, lung, or kidney and did not alter expression of IL-6 or IL-1β in any of the tissues studied (data not shown).

Role of Kupffer Cells in Temperature-Dependent Cytokine Expression

We previously reported that Kupffer cells were virtually the sole source of hepatic TNF-α and the predominant source of temperature-dependent plasma TNF-α generated in the LPS-challenged mouse (21). To determine if Kupffer cells were also essential for the enhanced IL-6 expression in the warmer mice, we analyzed plasma cytokine levels in temperature-clamped, LPS-challenged mice after depleting their Kupffer cells with intravenous liposome-encapsulated clodronate (31).

Fig. 1. Effects of temperature clamping on plasma tumor necrosis factor (TNF)-α and interleukin (IL)-6 concentrations in lipopolysaccharide (LPS)-challenged mice. A and D: kinetics of TNF-α (A) and IL-6 (D) expression. Groups of 6 CD-1 mice were temperature-clamped at 37 or 40°C and injected intraperitoneally with 50 µg LPS. Control (unclamped) mice were injected with LPS but remained conscious at normal ambient temperature (22–24°C). Groups of 6 mice were killed just before and 1, 2, 3, 4, or 5 h after LPS injection, and plasma TNF-α and IL-6 concentrations were measured by ELISA (means ± SE). *P < 0.02 vs. 37°C clamped and unclamped mice. ‡P < 0.02 vs. unclamped mice. §P < 0.001 vs. 37°C clamped and unclamped mice. B and E: LPS dose response for plasma TNF-α (B) and IL-6 (E) expression. Groups of 5 mice were temperature-clamped with the indicated dose of LPS, and killed 1 or 3 h later for quantitation of plasma TNF-α or IL-6, respectively. *P < 0.02 vs. 37°C mice. C and F: effects of intermediate temperatures on TNF-α (C) and IL-6 (F) expression. Groups of 5 mice were temperature clamped at the indicated temperature, injected intraperitoneally with 50 µg LPS, and killed 1 or 3 h later for quantitation of plasma TNF-α or IL-6, respectively. *P < 0.01 vs. 37°C mice at the same LPS dose.
depletion reduced the excess plasma TNF-α levels generated in the 40°C mice by 81% (Table 1). In contrast, plasma IL-6 levels were comparable in 37°C control and 37°C Kupffer cell-depleted mice, implicating sources other than Kupffer cells in the generation of circulating IL-6. However, the increase in plasma IL-6 levels that occurred in 40°C sham-depleted mice was abrogated in the Kupffer cell-depleted mice.

Effect of Temperature Clamping on Expression of HSP-72

We previously reported that the temperature threshold for modifying TNF-α expression in the RAW 264.7 mouse macrophage cell line is at least 2°C below the threshold for inducing the heat-shock response in vitro (15). To determine if the temperature threshold for modifying cytokine production (39–39.5°C) was distinct from the threshold for the heat-shock response in vivo, we measured HSP-72 in liver and kidney homogenates from temperature-clamped mice by immunoblot analysis (Fig. 4). Temperature clamping at 39.5°C for 3 h (Fig. 4, lanes 3 and 4) increased hepatic and renal HSP-72 levels compared with 37°C mice (lanes 1 and 2), but HSP-72 levels in the 39.5°C animals reached only one-half the levels achieved in the positive control mice warmed to 42°C for 20 min (lanes 5 and 6).

**DISCUSSION**

In this study, we showed that an increase of 2–2.5°C above basal core temperature and within the murine febrile range (18) profoundly altered the magnitude, kinetics, and tissue distribution of TNF-α, IL-6, and IL-1β in LPS-challenged mice. These changes included:

1) earlier and higher peaks in plasma and liver TNF-α levels;
2) higher circulating and tissue-associated IL-6 levels;
3) attenuation of the early plasma IL-1β peak and a delay in the late peak, preventing simultaneous exposure to TNF-α and IL-1β; and
4) enhanced pulmonary expression of IL-1β.

Mice have similar basal and febrile temperatures as humans (18), but their partially ectothermic nature (2) makes them particularly useful for studies requiring manipulation of core temperature. Armstrong (4) and Bell and Moore (6) demonstrated that murine core temperature could be maintained ~2°C above basal levels for several days by housing conscious mice in a warm environment. In the present study, we used an
EFFECTS OF CORE TEMPERATURE ON CYTOKINE EXPRESSION

Table 1. Effect of Kupffer cell depletion on temperature-dependent, LPS-induced plasma cytokine levels

<table>
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<tr>
<th></th>
<th>TNF-α, pg/ml</th>
<th>IL-6, ng/ml</th>
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<tr>
<td></td>
<td>37°C</td>
<td>40°C</td>
</tr>
<tr>
<td>Control</td>
<td>1,480 ± 484</td>
<td>4,882 ± 1,190*</td>
</tr>
<tr>
<td>Kupffer cell depleted</td>
<td>131 ± 39*</td>
<td>779 ± 194‡</td>
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Cytokine levels were measured by ELISA and are expressed as means ± SE. Kupffer cells were depleted in groups of 4 mice by administering a single 0.1-ml iv dose of liposome-encapsulated clodronate 2 days before lipopolysaccharide (LPS) challenge. Control mice received 0.1 ml of saline. Mice were temperature clamped at 37 or 40°C, injected intraperitoneally with 50 µg LPS, and killed 1 or 3 h later for measurement of plasma tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 levels, respectively. *P < 0.05 compared with 37°C control mice. †P < 0.05 compared with 37°C Kupffer cell-depleted mice. ‡P < 0.05 vs. 40°C control mice.

We previously showed that Kupffer cells were the major source of circulating TNF-α in 37°C clamped, LPS-challenged mice, as well as the source of the excess circulating TNF-α present in the 40°C animals (21). In this study, we found that, while enhanced TNF-α levels were restricted to the liver in the 40°C mice, IL-6 levels were severalfold higher in most tissues studied in the warmer animals. Thus it was not surprising that Kupffer cell depletion, which markedly attenuated TNF-α generation, did not reduce plasma IL-6 levels in LPS-challenged 37°C mice. However, the increase in plasma IL-6 levels that occurred in 40°C mice was abrogated by prior Kupffer cell depletion. We have considered two possible explanations for the loss of excess IL-6 generation in the Kupffer cell-depleted 40°C mice. The Kupffer cell may be the predominant source of the excess IL-6 generation in the febrile mouse. Although IL-6 levels increased in most tissues studied in the 40°C mice, the greatest relative increase in IL-6 expression occurred in the livers of the warmer animals. Alternatively, Kupffer cells may release a factor that stimulates a widespread increase in IL-6 expression in the warmer animals. TNF-α is the most obvious candidate for this Kupffer cell-generated, IL-6-inducing factor. TNF-α is a potent inducer of IL-6 expression (28), and circulating levels of TNF-α were greatly reduced in the Kupffer cell-depleted mice. However, IL-6 expression at 37°C was comparable in the control and Kupffer cell-depleted mice even though circulating TNF-α levels were reduced by 87% in the Kupffer cell-depleted animals. Furthermore, LPS can directly induce IL-6 expression in the absence of TNF-α (3). It may also be possible that tissues are more responsive to the IL-6-stimulating action of TNF-α in anesthetized temperature-clamping model rather than conscious mice for the following reasons. First, this was a short-term study focused on events occurring within 5 h of LPS challenge, during which anaesthesia could be safely maintained. Second, the use of anaesthesia avoided the potentially profound immunomodulatory effects of physical stress imposed by handling and LPS challenge (22). Third, anaesthesia and immersion in water baths provided much more rapid and precise control of core temperature than is possible in conscious mice. This model was intended to isolate the effects of the core temperature increase from the other components of the febrile response, and our results should be interpreted in this context. Although anaesthesia itself may potentially disturb the normal immune responses (9), we previously showed that comparable increases in core temperature caused similar changes in LPS-induced TNF-α expression in anesthetized and conscious mice (21). In the present study, circulating and organ-associated cytokine levels after LPS challenge were similar in the anesthetized 37°C mice and in the conscious control animals, which maintained similar core temperatures. These data do not exclude immuno-suppressive effects of some anesthetic agents but indicate that the anesthetic protocol used in this study did not significantly affect the measured generation of cytokines.

Three previous studies have reported that raising core temperature in rodents either reduced (11, 22) or had no effect (7) on circulating TNF-α and IL-1 expression. However, in these studies the animals were warmed to temperatures above the normal febrile range and within the heat-shock range (≥41°C). Furthermore, the core temperature increase in these studies either preceded LPS challenge by 6–7 h (7) or 24 h (22) or followed LPS challenge (11). We previously reported that the core temperature increase must occur before or coincident with LPS challenge to enhance early TNF-α expression (21). Delaying the increase in core temperature for 30 min after injection of LPS abrogated its effects on TNF-α expression. Taken together, these data suggest that the effect of increasing core temperature on TNF-α expression is determined by both the magnitude of the temperature increase and the timing of the core temperature change relative to challenge with an injurious stimulus. In the present study, mice were challenged with LPS immediately after target core temperature was reached. This schedule models recurrent endotoxemia and bacteremia occurring in the setting of fever, which often occurs during serious bacterial infections (29).

We previously showed that Kupffer cells were the major source of circulating TNF-α in 37°C clamped, LPS-challenged mice, as well as the source of the excess circulating TNF-α present in the 40°C animals (21). In this study, we found that, while enhanced TNF-α levels were restricted to the liver in the 40°C mice, IL-6 levels were severalfold higher in most tissues studied in the warmer animals. Thus it was not surprising that Kupffer cell depletion, which markedly attenuated TNF-α generation, did not reduce plasma IL-6 levels in LPS-challenged 37°C mice. However, the increase in plasma IL-6 levels that occurred in 40°C mice was abrogated by prior Kupffer cell depletion. We have considered two possible explanations for the loss of excess IL-6 generation in the Kupffer cell-depleted 40°C mice. The Kupffer cell may be the predominant source of the excess IL-6 generation in the febrile mouse. Although IL-6 levels increased in most tissues studied in the 40°C mice, the greatest relative increase in IL-6 expression occurred in the livers of the warmer animals. Alternatively, Kupffer cells may release a factor that stimulates a widespread increase in IL-6 expression in the warmer animals. TNF-α is the most obvious candidate for this Kupffer cell-generated, IL-6-inducing factor. TNF-α is a potent inducer of IL-6 expression (28), and circulating levels of TNF-α were greatly reduced in the Kupffer cell-depleted mice. However, IL-6 expression at 37°C was comparable in the control and Kupffer cell-depleted mice even though circulating TNF-α levels were reduced by 87% in the Kupffer cell-depleted animals. Furthermore, LPS can directly induce IL-6 expression in the absence of TNF-α (3). It may also be possible that tissues are more responsive to the IL-6-stimulating action of TNF-α in
the warmer animals. We are presently using a murine TNF-α knockout model to determine the role of TNF-α in mediating the enhanced IL-6 expression that occurs in warmer animals.

In this study, a core temperature increase of 2–2.5°C above basal temperature was sufficient to modify the generation of acute-phase cytokines. Increases in core temperature of this magnitude commonly occur during infections as well as during vigorous physical exercise, in response to stress, and as a result of exposure to high ambient temperatures in many species, including humans and mice (24). We previously reported that the temperature threshold for modifying TNF-α expression in vitro was lower than that for inducing heat shock in cultured macrophages (15, 16). In this study, we found that the temperature threshold for modulating cytokine generation in vivo was only sufficient to induce submaximal HSP-72 expression in kidney and liver, suggesting the two processes are likely to be distinct.

Survival of the infected host depends on a delicate balance between enhanced antimicrobial host defense mechanisms and collateral tissue injury (12). We previously showed that an elevation in core temperature such as occurs in fever may tip the balance toward enhanced antimicrobial defense by amplifying expression of the proinflammatory cytokine TNF-α (21). In this study, we showed that fever exerts effects on cytokine expression that may also reduce the risk of host injury. First, while fever may enhance early TNF-α expression, it limits its toxicity by reducing the duration of its expression and preventing simultaneous expression of TNF-α and IL-1β (34). Second, fever caused enhanced generation of IL-6 in most tissues. Although IL-6 enhances the acute-phase response (17), we previously reported that the temperature threshold for modifying IL-6 expression in most tissues.

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