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RESEARCH ARTICLE

Prolonged exposure to hyperthermic stress augments neutrophil recruitment to lung during the post-exposure recovery period

MOHAN E. TULAPURKAR[1], JEFFREY D. HASDAY[1,2,3,4], & ISHWAR S. SINGH[1,2,4]

[1]*Division of Pulmonary and Critical Care, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland,* [2]*Mucosal Biology Research Center, University of Maryland School of Medicine, Baltimore, Maryland,* [3]*Cytokine Core Laboratory, University of Maryland School of Medicine, Baltimore, Maryland, and* [4]*Research Services of the Baltimore VA Medical Center, Baltimore, Maryland, USA*

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Abstract

The effects of heat, especially long-term heat exposure, are complex and incompletely understood and few studies have analysed the immunological consequences of such exposures. In the present study we analysed how long-term hyperthermia modified the pulmonary immune responses, especially recruitment of neutrophils to sites of inflammation, infection and injury. Using our mouse model of long-term whole body hyperthermia (continuous 5-day passive febrile range hyperthermia (5d-FRH)) we found that bacterial lipopolysaccharide (LPS) challenge greatly increased neutrophil accumulation in bronchoalveolar lavage and lung parenchyma in 5d-FRH exposed mice in comparison to LPS-treated controls. Moreover, the effect was sustained, and persisted during the post-exposure recovery period, and LPS challenge on days 5–7 post-recovery also exhibited similarly augmented neutrophil response. Lung lavage from 5d-FRH mice, either immediately or up to 7 days post-exposure, showed significantly increased levels of ELR + CXC chemokines, KC or LIX in response to LPS challenge, indicating that enhanced chemokines could contribute to the increased recruitment of neutrophils to the lung. However, an in vivo neutrophil migration assay following 5d-FRH and during the post-exposure recovery period also showed persistently enhanced neutrophil influx in response to a fixed chemotactic gradient generated by recombinant human IL-8, suggesting that additional mechanisms besides increased ELR + CXC chemokines contributed to the augmented neutrophil response caused by 5d-FRH exposure. These previously unappreciated profound and lasting effects of long-term hyperthermia may have important consequences and may help explain the increased risk of respiratory illnesses in active duty personnel and returning veterans.

Keywords: *Hyperthermia, long-term stress, neutrophil, LPS, chemokines*

Introduction

The effects of heat stress are complex, profound, and incompletely understood. Acute exposure to heat shock or mild whole body hyperthermia elicits a myriad of effects, including activation of the heat shock response and induction of heat shock protein (HSP) genes [1–3]. Several studies, including those from our group, have indicated that heat shock and/or hyperthermia can also modify host immune responses including cytokine-chemokine gene expression and mobilisation of lymphocytes and neutrophils [4–16].

We have shown that exposing mice to whole body hyperthermia in the febrile range (febrile-range hyperthermia(FRH) core temperature ~39.5°C) augments recruitment of neutrophils to sites of infection, inflammation, and injury via multiple converging mechanisms, including: (a) increased generation of chemokines and GM-CSF; (b) induction of G-CSF and expansion of the circulating neutrophil pool; and (c) increased endothelial capacity for neutrophil transendothelial migration [9–11, 17]. These actions of FRH greatly accelerate pathogen clearance but also increase collateral tissue

Correspondence: Ishwar S. Singh Phd, University of Maryland School of Medicine, Health Science Facility-II, Room S311, 20 Penn Street, Baltimore, MD 21201, USA. Tel: 410-706-5507. Fax: 410-706-5508. E-mail: isingh@umaryland.edu

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injury and the net outcome depends on the nature of the pathologic process and the tissue affected [9, 10, 18]. For example, FRH exposure improved survival in a mouse model of *Klebsiella pneumoniae* peritonitis [18] but worsened survival in the pneumonia model with the same pathogen [9] despite similar enhancement of pathogen clearance indicating increased vulnerability of the lung to collateral tissue injury caused by neutrophil-dependent inflammation. In fact, the observed occurrence of acute respiratory distress syndrome (ARDS) in almost a quarter of patients with acute heat stroke [19] and the increased susceptibility of the pulmonary epithelium to apoptosis following hyperthermia [20], provide further support that the lung is at relatively increased risk of injury in the setting of hyperthermia.

Compared with the acute effects of short-term heat exposure, little is known about chronic exposure to hyperthermia. Although long-term heat exposure results in heat acclimation (HA) and acquired thermal tolerance (ATT) [21, 22], the immunological consequences of such exposure are poorly understood. A survey study of troops participating in Operation Bright Star in Egypt showed 47% of 1454 deployed troops reported a respiratory illness [23]. A survey of returning troops participating in Operation Enduring Freedom/Operation Iraqi Freedom showed both asthmatic and non-asthmatic participants had increased respiratory symptoms of wheezing, cough, sputum production, chest pain/tightness, and allergy symptoms during deployment compared to pre-deployment [24]. These studies not only indicate that long-term hyperthermia exerts important immunomodulatory effects but also implicate the vulnerability of the lung and the respiratory tract to its adverse effects.

In the present study we used our recently developed mouse model of long-term hyperthermia comprising a continuous 5-day exposure to passive FRH (5d-FRH) [22] and a well-characterised model of lung inflammation induced by endotoxin inhalation [5, 9] to determine the consequences of long-term hyperthermia on regulation of inflammation in the lung. We found that such exposure caused a persistent alteration in the lung immune response characterised by a greatly increased capacity for bacterial lipopolysaccharide (LPS)-induced neutrophil accumulation in bronchoalveolar lavage fluid (BALF) and lung parenchyma, in part through increased expression of ELR + CXC chemokines. Moreover, the effects were sustained and lasted for several days (5–7 days) during the recovery period after cessation of hyperthermia exposure. These previously unappreciated profound and lasting effects of long-term hyperthermia may have important consequences and may help explain the increased risk of respiratory illnesses in active duty personnel and returning veterans.

Materials and methods

5d-FRH exposure

Male CD-1 mice weighing 30–35 g were purchased from Charles River (Wilmington, MA) and housed in the Animal Care Facility in the Veteran's Administration Medical Center, Baltimore, under Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved conditions and under the supervision of a full-time veterinarian. Mice were adapted to standard plastic cages for at least 4 days before the study and were used within 4 weeks of arrival. To avoid the influence of diurnal cycling, all experiments were started at approximately the same time each day (between 8:00 and 10:00 AM). Mice were implanted with intraperitoneal telemetric thermistors (Data Safety International; St. Paul, MN; ETA-F10) 10 days prior to FRH exposure. A sterilised ETA-F10 transmitter was placed into the peritoneal cavity and subcutaneous electrodes secured under isoflurane anaesthesia as described in the manufacturer's protocol. The mice received 0.1 mg/kg buprenorphine analgesia subcutaneously every 12 hours (s.c. q12h) for 2 postoperative days, housed one mouse per cage and were provided with food and water ad libitum immediately after surgery and allowed to recover for 10 days at 24–25°C ambient temperature. FRH was imposed by transferring the mice in the standard cages into modified Air Shields^{TM} infant incubators containing DSI data receivers with air temperature set to 37°C and core temperature monitored using the DSI Automated Data Acquisition System for 5 days (5d-FRH). Normothermic controls were housed at standard room temperature (24–25°C). Following the 5d-FRH-exposure protocol, mice were immediately (0d post-5d-FRH) challenged with intra-tracheal (i.t.) LPS or IL-8 (see below) or were allowed to recover at standard room temperature for up to 7 days prior to LPS or IL-8 challenge. Except for the ambient temperature, handling of normothermic control and 5d-FRH mice was identical. All procedures were approved by the Baltimore VA and the University of Maryland, Baltimore, Animal Care and Use Committee.

Intra-tracheal LPS/IL-8 instillation

Mice were challenged with LPS (50 μg LPS in 50 μL sterile PBS) or with 50 μL sterile PBS i.t. as described before [5, 8, 9] either immediately (0 days post-exposure recovery) or during the

post-exposure recovery period as indicated. All LPS instillations were for 24 h at standard room temperature following which the mice were euthanised and lungs were either lavaged for analysis of cell and cytokine composition or inflation-fixed for confocal immunofluorescence analysis as described earlier [5, 8–10].

To analyse how FRH modified the in vivo capacity for chemokine-directed trans-alveolar migration (TAM) of neutrophils, mice were challenged with 1 μg recombinant human IL-8 (rhIL8) (R&D Systems, Minneapolis, MN) in 50 μL sterile PBS using the same protocol as for LPS, except the mice were euthanised 4 h later at room temperature and analysed for lung lavage composition as above.

Bronchoalveolar lavage fluid (BALF) cell and cytokine composition

Cells were collected by centrifugation and total cell counts and differential cell counts of Diff-Quick^{TM}-stained cytopreparations were performed manually using a haemocytometer by two blinded observers using morphologic criteria as described earlier [9, 10]. Mouse KC/CXCL1, MIP-2/CXCL2, and LIX/CXCL5 were measured by ELISA in the cell-free supernatants as we have previously described [5, 8–10].

Immunofluorescence confocal microscopy

After euthanasia, the anterior chest wall was resected, the trachea cannulated with an 18-gauge blunt needle, and the lungs were inflated *in situ* with 4% v/v paraformaldehyde at 20 cm H_2O pressure. The trachea and lungs were embedded *en bloc* in paraffin and 25 μm sections were cut immunostained with biotinylated rat anti-mouse Gr-1 (AbD Serotec, Oxford) to identify neutrophils and rabbit anti-VE-cadherin (Sigma, St Louis, MO) to identify endothelium. Non-specific signal was reduced by sequentially blocking with 1% donkey serum (v/v) in PBST for 30 min and with a commercial avidin/biotin blocking kit (Vector, Burlingame, CA) according to the manufacturer's instructions, then sequentially incubated overnight with primary antibody and a 1:500 dilution of appropriate secondary antibody for 1 h, and mounted. The immunostained sections were visualised using an Olympus microscope and Fluo View confocal software (Olympus America, Center Valley, PA). 1024 × 1024-pixel z series images (1 uM step size) for each fluorophore were obtained. The number and localisation of the neutrophils was analysed using the Neurolucida^{TM} software. The localisation of the neutrophils relative to the vascular endothelium was determined using a modification of the criteria described by Woodfin et al. [25]. In brief, if endothelial staining was observed on both sides of the long axis of a neutrophil, the cell was classified as intravascular. If endothelial staining was absent from either side of a neutrophil, it was classified as extravascular or extravasating.

Data analysis

Data are presented as mean ± SE. Differences between more than two groups were analysed by one-way analysis of variance (ANOVA); post-hoc analysis was conducted using the Tukey honestly significant difference (HSD) test.

Results

Effect of ambient temperature on core temperature of mice exposed to FRH

Core temperature of FRH-exposed and normothermic controls was remotely monitored in at least one mouse per group using the Data Sciences International Automated Data Acquisition System (St Paul, MN) every 20 s for 120 h and hourly averages were calculated. The core temperature exhibited a circadian pattern in both control and FRH exposed mice (Figure 1), but core temperature in the FRH mice was approximately 2°C higher than the normothermic controls and this difference was maintained for the entire 5-day exposure. Upon removal to room temperature after 5d-FRH, core temperature of the mice returned to baseline levels within 60–90 min (data not shown) [22].

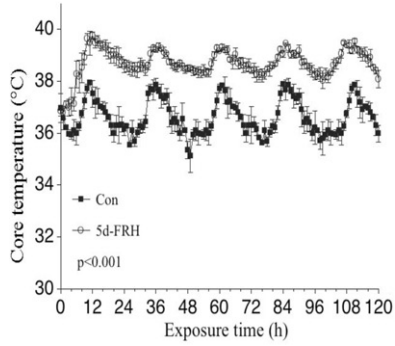

Figure 1. Core temperature during passive 5d-FRH: Mice implanted with i.p. sensors were housed at 25°C (Control) or 37°C (FRH) ambient temperature for 5 days while continuously monitoring core temperature. The mean temperature for each hour was calculated. Two experiments, each with four mice per group, were pooled. Data are mean ± SE. Core temperature in FRH mice was greater than control with $p < 0.001$ by repeated measures ANOVA.

5d-FRH markedly enhanced neutrophil recruitment to the lung following i.t. LPS challenge

We have earlier shown that exposure to acute FRH for 24 h augmented neutrophil accumulation in BALF following i.t. LPS [9]. To determine whether 5d-FRH had similar effects, we determined neutrophil content of BALF collected 24 h after i.t. LPS in 5d-FRH exposed and normothermic control mice (Figure 2A). As previously reported, i.t. LPS for 24 h caused enhanced neutrophil accumulation in BALF, but the effect was markedly increased by approximately 2-fold in the 5d-FRH exposed group (2.55×10^6 versuss $\sim 1.32 \times 10^6$, $p < 0.001$). More interestingly, the effect of 5d-FRH on LPS-induced BALF neutrophilia persisted for several days after cessation of 5d-FRH exposure and mice challenged with LPS for 24 h even on day 2, day 3 or day 5 post-5d-FRH showed significantly higher count of neutrophils in the BALF (3.72×10^6, 2.49×10^6 and 2.38×10^6, respectively, $p < 0.05$ with respect to LPS-challenged normothermic controls).

To further analyse the effects of 5d-FRH and recovery on neutrophil recruitment from the pulmonary microvasculature, normothermic and 5d-FRH-exposed mice were treated with or without i.t. LPS for 24 h and lungs were inflation-fixed, immunostained with antibodies against Gr-1 and VE-cadherin to identify neutrophils and endothelium, respectively, and analysed by immunofluorescence confocal microscopy (Figure 2B). Prior to i.t. LPS instillation, neutrophil sequestration in the pulmonary vasculature was substantially higher in the 5d-FRH mice compared with normothermic controls (Figure 2B, compare panel 0- with C-) and the effect persisted for at least 5 days post-5d-FRH exposure (Figure 2B, compare panels 5- and 7- with 0-). In normothermic mice, LPS instillation also increased neutrophil counts in the pulmonary microvasculature but the effect was modest (Figure 2B, compare panels C− with C+) and not as distinct as observed with BALF analysis (Figure 2A). However, similar to BALF analysis, LPS instillation greatly enhanced neutrophil accumulation in the terminal airspaces in 5d-FRH-exposed mice (Figure 2B, compare panels 0+ with C+) and the effects paralleled neutrophil sequestration during the post-exposure recovery period (Figure 2B, panels 3+, 5+ and 7+).

To further define the effect of 5d-FRH exposure and recovery on neutrophil recruitment in lung, we quantified neutrophil intravascular retention and extravasation in the confocal images using a previously described protocol [25] (Figure 2C). Neutrophils were classified as intravascular if they were completely contained within the blood vessel. Otherwise they were classified as extravasating/extravascular. This analysis further showed that the neutrophil content in lung was increased several fold following 5d-FRH, that ~80% of these cells were intravascular, and that this effect lasted for at least 5–7 days after 5d-FRH exposure. In control mice, i.t. LPS caused a modest increase in the total neutrophil content but, most importantly, increased the proportion of total neutrophils that had extravasated from ~0% to 50%. 5d-FRH exposure markedly increased the total number of neutrophils in the lung by several fold till day 5–7 of recovery but the proportion of extravasated neutrophils was around 20% in the absence of LPS challenge. LPS instillation further enhanced the lung neutrophil content by approximately two-fold and also enhanced the extravascular or extravasating proportion to about 75% which was comparable throughout the recovery period.

5d-FRH-exposure greatly enhanced LPS-induced CXC chemokine levels in the BALF

We have previously shown that FRH concurrent with i.t. LPS [8, 9] or hyperoxia [10] augmented CXC chemokine levels in BALF and that this effect is dependent upon the stress-activated transcription factor heat shock factor-1 (HSF1) [8, 26, 27]. To analyse the effect 5d-FRH exposure and its potential contribution to the incremental neutrophil accumulation, we measured endogenous ELR + CXC chemokines, KC (CXCL1), MIP-2 (CXCL2), and LIX (CXCL5) levels in BALF of control and 5d-FRH exposed mice 24 h after i.t. LPS instillation (Figure 3). As expected, LPS instillation increased BALF levels of all three ELR + CXC chemokines in controls and in 5d-FRH-exposed mice the levels of KC and LIX (but not MIP-2) were increased further by 1.6- and 1.9-fold, respectively. Furthermore, the effect of 5d-FRH exposure on LIX expression persisted throughout the 7-day recovery period, whereas, LPS-induced KC expression returned to pre-5d-FRH-exposure levels by the third day of recovery. In the absence of i.t. LPS, 5d-FRH-exposed and control mice had similarly low BALF levels of the three ELR + CXC chemokines (data not shown).

IL8-directed transalveolar migration of neutrophils is also enhanced after 5d-FRH

Using an in vivo IL-8-directed neutrophil TAM assay we have recently found that FRH for 16–24 h profoundly augmented subsequent accumulation of neutrophils in BALF after i.t. IL-8 [28]. Using the same assay we found that 1 μg rhIL-8 i.t. caused about 3.3-fold more neutrophil accumulation in the

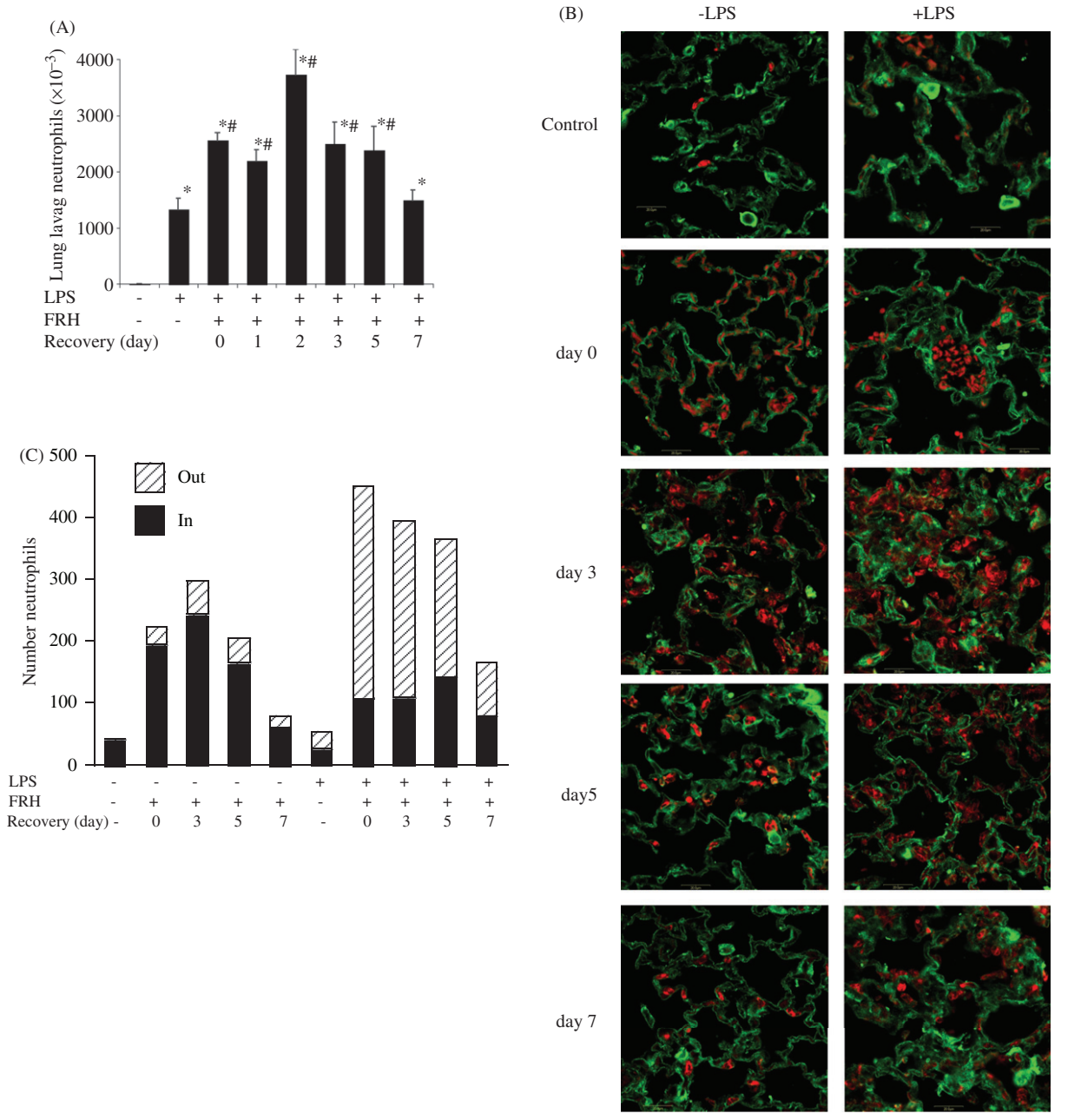

Figure 2. 5d-FRH exposure increases neutrophil recruitment to lung after i.t. LPS challenge. Mice were exposed to 5d-FRH and then transferred to room temperature for post exposure recovery. LPS-treated normothermic controls or 5d-FRH-exposed mice received 50 μg LPS i.t. either immediately (0 days) or as indicated whereas LPS-untreated mice received sterile PBS and euthanised after 24 h at room temperature. (A) lungs were lavaged and total neutrophil content determined by manual counting. Mean ± SE of 4 mice per group. * and # denotes $p < 0.05$ versus PBS- or LPS-treated controls, respectively. (B) lungs were inflation/fixed, stained for neutrophils (Gr-1, red) and endothelium (VE-cadherin, green) and analysed by confocal microscopy. Representative fields of three mice per group are shown. (C) Confocal images were analysed for number of total, intravascular (IN) and extravasating (OUT) neutrophils per 60× field. Mean of three fields per mouse, three mice per group plotted.

BALF of 5d-FRH mice than in normothermic mice ($\sim 245 \times 10^3$ versus 74×10^3 cells/mL, $p < 0.001$) (Figure 4). Moreover, the effect persisted during the recovery period and although it waned during recovery, it was still significantly greater after 7 days of post-5d-FRH exposure. Most importantly, i.t. rhIL-8 for 4 h did not alter BALF levels of KC, LIX, or MIP-2 in controls or 5d-FRH exposed mice (data not shown) suggesting the enhanced recruitment of neutrophils after 5d-FRH was not only due to enhanced LIX and KC levels but also due to other contributing mechanisms.

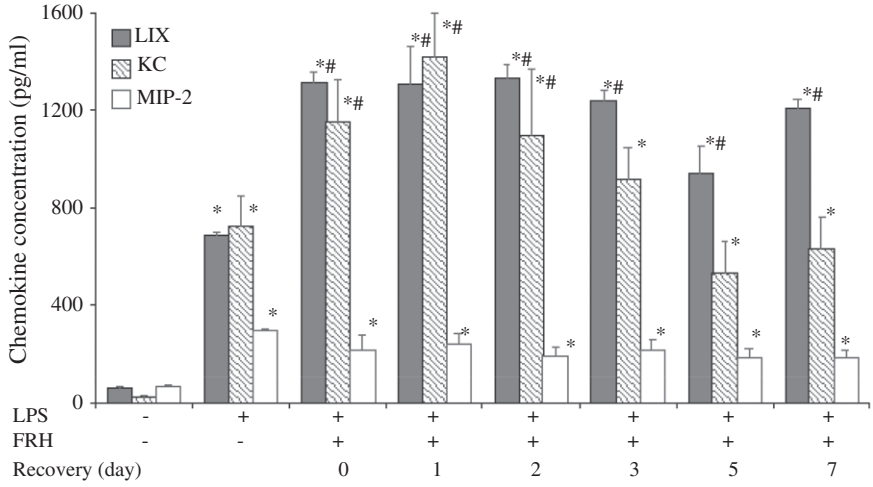

Figure 3. 5d-FRH exposure has a profound and lasting effect on LPS-induced CXC chemokine levels in lung: Mice were treated as indicated above (Figure 2), lungs lavaged, and KC, LIX and MIP-2 assayed by ELISA. Data are mean + SE, of four to six mice per group. * and # denotes $p < 0.05$ versus PBS or LPS-treated controls, respectively.

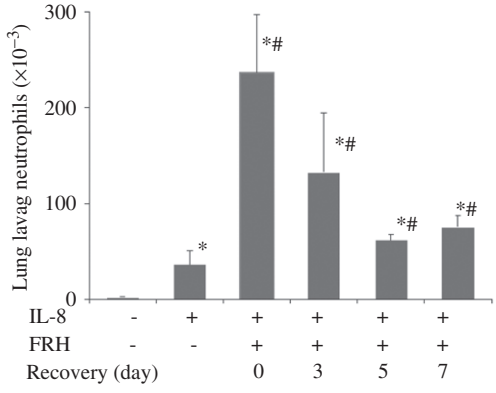

Figure 4. 5d-FRH exposure increases capacity for IL-8-directed neutrophil transalveolar migration. Mice were treated as indicated above (Figure 2) except 1 μg rhIL-8 was instilled i.t. instead of LPS and mice were euthanised for lung lavage 4 h later and neutrophils counted. Mean ± SE of four mice per group. * and # denotes $p < 0.05$ versus PBS- or LPS-treated controls, respectively.

Discussion

Neutrophils are among the earliest leukocyte responders to infection, inflammation, and injury and play an essential role in containing infections through release of cytotoxic effector molecules [29, 30]. Unfortunately, the same effector molecules can also cause substantial collateral tissue injury, especially to highly susceptible sites such as the lungs [31]. Neutrophil accumulation and enhanced neutrophilia in BALF has been correlated with poor prognosis in septic ARDS and acute lung injury (ALI) and neutrophil-mediated cytotoxicity has been cited as the major contributor to ALI and ARDS [32–35].

Our group has previously shown that acute, concurrent exposure to FRH exerts multiple actions that profoundly increase neutrophil recruitment, enhance pathogen clearance and augment lethal lung injury in mouse models of pneumonia and hyperoxia [9, 10]. We showed that acute FRH exposure converted non-lethal lung inflammation induced by i.t. instillation of LPS in normothermic mice to a lethal, neutrophil-dependent lung injury [9] suggesting that the increase in core temperature that occurs during febrile illnesses can increase collateral tissue injury in susceptible tissues, such as in the lung. In contrast, long-term heat exposure initiates adaptive changes including ATT and HA, a process of adaptive physiological changes that abates physiological strain, sustains physical and cognitive performance capabilities and protects against severe heat-related illnesses [21, 36, 37]. In a mouse model we showed that following exposure to 5d-FRH mice exhibited improved heat elimination and exercise tolerance during subsequent heat exposure and increased basal HSP72 expression [22], similar to what we observed with human HA [21].

In the present study we extend our previous studies of 5d-FRH and the immunological consequences of concurrent FRH by showing that exposing mice to a chronic hyperthermia protocol that mimics human HA exhibit greatly enhanced neutrophil accumulation in response to a proinflammatory stimuli. Our present results indicate that 5d-FRH can have a persistent and sustained alteration in the lung immune response by profoundly enhancing the number of neutrophils in the pulmonary vasculature as well as their ability to migrate to sites of infection and injury. Using two independent methods, cell count in the BALF (Figure 2A) and by confocal

microscopy of lung tissue sections (Figure 2B) we have shown that 5d-FRH markedly enhanced neutrophil recruitment to the lung and the influx was further augmented in response to a proinflammatory stimulus. Reutersham et al. [38] showed that neutrophil recruitment following LPS inhalation occurs through a stepwise process, beginning with increased neutrophil sequestration within the lung vasculature that is detectable within the first hour after LPS instillation and culminating in accumulation of neutrophils in the bronchoalveolar space by 12 to 24 h post-LPS. Confocal imaging demonstrated that mice exposed to 5d-FRH exhibited greatly increased neutrophil sequestration within the lung vasculature (Figure 2B, day 0), and by 24 h after LPS instillation, the total neutrophil content in lung doubled but the number of neutrophils remaining in lung vasculature had decreased by over half (Figure 2B, day 0+LPS and Figure 2C). Furthermore, mice exposed to 5d-FRH and allowed to recover for 3 or 5 days exhibited similar patterns of neutrophil sequestration and migration (Figure 2B and C) suggesting that the effect was persistent and lasted for several days after cessation of FRH exposure. This augmented neutrophil recruitment was accompanied with an increase in ELR^+ CXC chemokine LIX and KC levels in the BALF (Figure 3) indicating that an enhanced chemotactic gradient could be the contributing factor. However, neutrophil influx in the lung was increased in the 5d-FRH-exposed mice even in the absence of LPS challenge and increased CXC chemokine expression suggesting that additional mechanisms might also contribute to the process. To test this possibility we performed an in vivo IL-8-directed neutrophil TAM assay and found that neutrophil influx in the lung against a fixed chemotactic gradient generated with 1 μg rhIL-8 i.t. was significantly higher in 5d-FRH mice than normothermic controls (Figure 4) and similar to LPS instillation, the effect was persistent during the post-exposure recovery period. These studies indicate that in addition to increased CXC chemokine levels, other mechanisms involving both the neutrophils and the vascular endothelia might contribute to the enhanced neutrophil influx in the lung of 5d-FRH-exposed mice.

Our present results are unique and novel in two respects: first, no earlier study has demonstrated such a profound effect of hyperthermia on neutrophil recruitment to the pulmonary vasculature, and second, the persistent and lasting nature of the effect that could modify neutrophil priming and recruitment 5-7 days after the exposure. The fact that the effect was persistent and lasted for several days before returning or 'resetting' to normal baseline indicated that although reversible, the recovery after 5d-FRH was slow and prolonged during which the host displayed an altered inflammatory response to proinflammatory agonists. Enhanced neutrophil recruitment and neutrophil-mediated inflammation, loss of endothelial barrier function and epithelial injury are considered major contributors of ARDS and acute lung injury [39, 40]. It is likely that the increased incidences of multi-symptom illnesses [41–44], immune abnormalities [45–48], and respiratory illness including bronchitis and asthma [23, 24, 49, 50] reported in military personnel and returning veterans deployed in high temperature environments like the Gulf region might be due to environmental/exertional hyperthermia endured during deployment. The present study uniquely demonstrates that exposure to long-term hyperthermia can have profound and persistent consequences for innate immune function and regulation of inflammation, but the full spectrum of immunologic effects and mechanisms responsible remain to be elucidated. The importance of understanding the potential health effects of chronic exposure to hyperthermia and its long-term consequences is underscored by its relevance to large numbers of individuals who are exposed to environmental/exertional hyperthermia, including athletes, deployed troops and veterans.

Conclusion

Our studies show that 5d-FRH has a profound and distinct effect on neutrophil recruitment to the lung in response to a proinflammatory agonist. Furthermore, the effects are persistent and require several days before 'resetting' to normal during which the host displays a dysregulated response to proinflammatory agonists. Thus, long-term heat exposure that induces ATT and HA and protects against subsequent stress can be maladaptive in certain clinical contexts including infection, injury, and inflammatory disorders. Our studies underscore the complex effect of hyperthermia on host responses and the importance of understanding the immunological consequences especially on subsequent immune surveillance and/or sensitivity to infections and inflammatory disorders.

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