Death receptors mediate the adverse effects of febrile-range hyperthermia on the outcome of lipopolysaccharide-induced lung injury

Anne B. Lipke,1 Gustavo Matute-Bello,1,2 Raquel Herrero,3 Venus A. Wong,3 Stephen M. Mongovin,3 and Thomas R. Martin1,3

1Division of Pulmonary and Critical Care Medicine, 2Center for Lung Biology, and the 3Medical Research Service of VA Puget Sound Medical Center, University of Washington, Seattle, Washington

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Lipke AB, Matute-Bello G, Herrero R, Wong VA, Mongovin SM, Martin TR. Death receptors mediate the adverse effects of febrile-range hyperthermia on the outcome of lipopolysaccharide-induced lung injury. Am J Physiol Lung Cell Mol Physiol 301: L60–L70, 2011. First published April 22, 2011; doi:10.1152/ajplung.00314.2010.—We have shown that febrile-range hyperthermia enhances lung injury and mortality in mice exposed to inhaled LPS and is associated with increased TNF-α receptor activity, suppression of NF-κB activity in vitro, and increased apoptosis of alveolar epithelial cells in vivo. We hypothesized that hyperthermia enhances lung injury and mortality in vivo by a mechanism dependent on TNF receptor signaling. To test this, we exposed mice lacking the TNF-receptor family members TNFR1/R2 or Fas (TNFFR1/R2−/− and lpr) to inhaled LPS with or without febrile-range hyperthermia. For comparison, we studied mice lacking IL-1 receptor activity (IL-1R−/−) to determine the role of inflammation on the effect of hyperthermia in vivo. TNFFR1/R2−/− and lpr mice were protected from augmented alveolar permeability and mortality associated with hyperthermia, whereas IL-1R−/− mice were susceptible to augmented alveolar permeability but protected from mortality associated with hyperthermia. Hyperthermia decreased pulmonary concentrations of TNF-α and keratinocyte-derived chemokine after LPS in C57BL/6 mice and did not affect pulmonary inflammation but enhanced circulating markers of oxidative injury and nitric oxide metabolites. The data suggest that hyperthermia enhances lung injury by a mechanism that requires death receptor activity and is not directly associated with changes in inflammation mediated by hyperthermia. In addition, hyperthermia appears to enhance mortality by generating a systemic inflammatory response and not by a mechanism directly associated with respiratory failure. Finally, we observed that exposure to febrile-range hyperthermia converts a modest, survivable model of lung injury into a fatal syndrome associated with oxidative and nitrosative stress, similar to the systemic inflammatory response syndrome.

acute lung injury; oxidative stress; death receptor; fever

Fever is common in critically ill patients and is associated with as much as a threefold increase in odds of dying in the medical intensive care unit (ICU) (4, 6, 12, 40, 63). The relationship of fever to increased ICU mortality is not fully understood. In animal models of end-organ damage associated with critical illness, febrile-range hyperthermia (hereafter referred to as fever) significantly potentiates experimental injury of the heart, brain, kidney, and lung and increases mortality (10, 16, 26, 29, 44, 67, 77–79). However, the mechanisms by which fever enhances organ injury and mortality remain uncertain.

Hyperthermia in the range of clinically observed fever, for example 39.5–40°C, is not directly cytotoxic but enhances cell death in lymphocytes, polymorphonuclear neutrophils (PMN) and carcinoma cells by enhancing apoptosis, a programmed pattern of cell death (11, 28, 36, 37, 43, 54–59, 76). We and others have found that fever enhances lung injury in experimental models of acute lung injury (ALI) (1, 29, 44, 67, 69). We recently reported that lung injury complicated by fever is associated with increases in markers of apoptosis in the alveolar epithelium and is attenuated by the administration of N-benzyloxy carbonyl-Val-Ala-Asp (zVAD), an inhibitor of the caspase cascade that mediates apoptosis, suggesting that apoptosis of the alveolar epithelium is important to augmented lung injury associated with fever (44, 45). Furthermore, in alveolar epithelial cell lines and in primary alveolar epithelial cells, we demonstrated that hyperthermia enhances apoptosis specifically through increased TNF-α receptor activity by a mechanism associated with suppression of NF-κB activity.

The TNF-receptor superfamily, also known as the “death receptor” family, is a group of receptors related by the expression of extracellular cysteine-rich repeats (32). Members of the death receptor family include the TNF-receptor family members TNFR1/R2, Fas, and the TRAIL receptors. Although all of the TNF receptor family members have the ability to activate pathways leading to apoptosis and to inflammation, each individual member appears to preferentially trigger either apoptosis or inflammation, and this “preference” may be changed by interaction with other molecules or environmental factors. This paradigm is best defined for TNF-α. For example, when TNF-α binds the TNF receptor, the life or death of the cell depends largely on the activity of NF-κB, an inflammatory transcription factor, and on the specific TNF receptor (8). Typically, the TNF receptor activates intracellular kinases and potently induces the activity of NF-κB. However, if NF-κB activity is blocked, the receptors recruit a group of intracellular protein known as a death initiating signal complex that activates a cascade of proteases that mediate apoptosis, also known as caspases. In these circumstances, the TNF receptor can also activate c-Jun-N-terminal kinase (JNK), which can directly activate caspase-mediated cell death through release of Smac/Diablo. The activity of other members of the death receptor family may also be subject to modification by the activity of NF-κB or by modification of intracellular proteins such as the Fas-associates protein with death domain, an intracellular adaptor protein that associates with the death receptors (39, 70, 74). Members of the death receptor family may be clinically relevant because they have been associated with inflammation and apoptosis in ALI (47, 64, 65). Recently, Farnand et al. (18) have described that Fas induces simultaneous activation of death receptor and NF-κB pathways.
inflammation and apoptosis in epithelial cells, and these pathways are independent of each other and of NF-κB.

In contrast to the death receptor family, interleukin-1β (IL-1β) is the prototypical pyrogen that is potently inflammatory but may also be associated with cell death. This inflammatory cytokine is produced by macrophages, is found in the bronchoalveolar fluid of patients with ALI, and is thought to contribute to the pathogenesis of ALI (22, 42, 66). Overexpression of human IL-1β in the lungs of rats induces a robust inflammatory response, tissue injury, and is associated with eventual pulmonary fibrosis (38). Furthermore, animals deficient in IL-1 receptor activity are less susceptible to experimental ventilator-induced alveolar permeability, and the administration of IL-1 receptor antagonists diminishes pulmonary inflammation and alveolar edema associated with ventilator-induced lung injury (21). IL-1β has been shown to cause DNA fragmentation and cell death, as can be seen in apoptosis, in rat pancreatic islet cells in vitro, and this phenomenon can be attenuated by the administration of nitric oxide synthase inhibitors (17). Verma et al. (71) reported that IL-1β causes death of human-derived pancreatic cells by activation of JNK. Thus, because IL-1β has never been identified to bind to death receptors and directly induce apoptosis, the literature suggests that IL-1β can indirectly induce apoptosis through secondary mediators generated during inflammation.

On the basis of our in vitro data that TNF-α receptor activity was specifically enhanced during mild hyperthermia, in this study we sought to determine the role of TNF-α in the augmented lung injury and mortality associated with fever in vivo. We hypothesized that TNF-α receptor activity is required for fever to augment lung injury and consequent mortality. We compared the effect of fever on lung injury caused by inhaled lipopolysaccharide (LPS) in animals lacking TNF receptor 1 and 2 or Fas to determine the role of two major representatives of the death receptor pathway. As a control for other pathways that may lead to apoptosis indirectly, and for the effect of inflammation alone, we used animals lacking IL-1 receptor activity. The data show that deficiency in activity of the death receptors TNFR1/R2 or Fas or in IL-1 receptor activity attenuates mortality associated with fever and lung injury by a mechanism that involves systemic oxidative and nitrosative stress. The data show that TNF receptor activity and Fas activity are required for the adverse effects of fever on lung injury but that IL-1 is not. Finally, the data show that exposure to hyperthermia converts a model of mild lung injury with no mortality into a severe systemic inflammatory response with high mortality.

MATERIALS AND METHODS

Animal protocols. The animal protocols were approved by the Animal Care Committee of the VA Puget Sound Medical Center and the University of Washington, Seattle, WA. We studied C57BL/6J mice, mice lacking TNFR1/R2 (B6.129S-Tnfrsf1aand7–/–, B6.129S-Tnfrsf1b/tm1Imx, Tnfrsf1aand7–/–), mice lacking functional Fas receptor (B6.MRL-Fas–/–), and mice lacking IL-1 receptor (B6.129S7-Il1r1mel1ms2 Lps–/–, 1Il1r1mel1ms2, 1IL-1R–/–). Male mice aged 6–8 wk (20–30 g) (Jackson Laboratories, Bar Harbor, ME) were anesthetized with 4% heparinized xyrene and the plasma fraction was stored in individual aliquots at −80°C. Immediately after death, the thorax was opened, and the left hilum was clamped. The left lung was removed, weighed, and placed in PBS or heparinized syringes, and the lung was stored at −80°C. At a later date, the lung was homogenized in sterile water, and aliquots were stored at −80°C. Bronchoalveolar lavage (BAL) was performed in the right lung with four 0.5-ml aliquots of 0.9% NaCl with 0.6 mM EDTA at 37°C. For histological processing, the right lung was fixed in 4% paraformaldehyde at 15 cm H2O transpulmonary pressure. An aliquot of BAL fluid was used immediately for cell counts and differential. The remaining BAL fluid was spun for 10 min at 200 g, 4°C and supernatants were stored at −80°C.

Experimental design. The main experimental variables were genetic deficiencies in death receptor or IL-1 receptor signaling, core body temperature, and duration of exposure after LPS instillation. We tested four types of mice, C57BL/6, TNFR1/R2–/–, lpr, and IL-1R–/–, one dose of LPS (50 μg), two core body temperatures, 37°C (euthermic) and 39.5–40°C (hyperthermic), and two exposure times (24 or 48 h). This resulted in the experimental groups shown in Table 1. Intratracheal instillation of PBS served as a negative control for LPS instillation.

Measurements. Alveolar permeability was assessed by measurement of BAL fluid total protein using the bicinchoninic method (BCA assay; Pierce, Rockford, IL) and BAL IgM concentrations using a commercial immunoassay (Bethyl Laboratories, Montgomery, TX). Total cell counts were performed on an aliquot of BAL fluid using a hemocytometer. Cell differentials were determined on cytospin preparations stained with a modified Wright-Giemsa stain (Diff-Quick;
Fisher Scientific, Kalamazoo, MI). Plasma malondialdehyde concentrations were measured using a thiobarbituric acid reactive substances assay (Cayman Chemical, Ann Arbor, MI). The plasma concentrations of nitric oxide metabolites were assessed using a colorimetric assay in which nitrate was first converted to nitrite (Cayman Chemical). Cytokine concentrations in lung homogenates were assessed by immunoassay using multiplex profiling reagents and a Luminex 200 analyzer (R&D, Minneapolis, MN). The lower limits of detection for the cytokines were: TNF-α, 7.13 pg/ml; IL-1β, 61.04 pg/ml; IL-6, 10.43 pg/ml; IL-10, 8.02 pg/ml; keratinocyte-derived chemokine (KC), 14.4 pg/ml; macrophage inflammatory protein-2 (MIP-2), 6.86 pg/ml; granulocyte/macrophage colony-stimulating factor (GM-CSF), 8.92 pg/ml.

Histology. After fixation, the lungs were embedded in paraffin, cut in 4-μm sections, and stained with hematoxylin and eosin. TUNEL assay was performed in paraffin-embedded lung tissue according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). Fluorescence microscopy was performed using a Nikon Eclipse 80i microscope (Nikon, Melville, NY). Lung injury scores were generated in a blinded manner using a quantitative injury score as previously described (48). To generate the lung injury score, 300 alveoli of lung tissue samples were examined at ×400 magnification. Each field was examined and assigned points according to predetermined criteria. Lung tissue samples from two mice in each treatment group were examined and their scores averaged to produce a final score for each treatment group. TUNEL-positive cells were quantified in a blinded manner on 10 randomly generative visual fields at ×100 magnification.

Statistics. The data were analyzed using GraphPad Prism 5.0 software (San Diego, CA). Comparisons of the means of more than two groups were performed using one-way ANOVA, and post hoc comparisons were performed using the Bonferroni-Dunn test. Comparisons between two groups were performed using an unpaired t-test. Survival curves were compared with a log-rank test. Data are shown in the figures as the mean with notation of the standard error in a bar. A P value <0.05 was considered significant.

RESULTS

Hyperthermia enhances mortality from intratracheal LPS by a mechanism requiring TNFR1/R2, Fas, and IL-1R. Mice deficient in TNFR1/R2, Fas, or IL-1 receptor were treated with 50 μg of intratracheal LPS and then maintained at normal temperature (euthermia) or ambient hyperthermia for up to 48 h. The responses of these mice to LPS and hyperthermia were compared with the response of C57BL/6 mice (wild-type, WT), which were similarly treated. Exposure to ambient hyperthermia resulted in an increase in core body temperature to 39.5–40°C in sentinel mice, which was maintained for the duration of the experiment (Fig. 1).

All of the animals treated with intratracheal PBS (n = 24) survived the duration of the experiment, independent of body temperature (data not shown). Similarly, all of the animals treated with LPS and euthermia (n = 24) survived for the duration of the experiment (Fig. 2, A–C). In contrast, WT mice exposed to LPS and hyperthermia had a mortality of 63.6 to 71.5% by 48 h (n = 21/30, P < 0.0001 compared with euthermic C57BL/6 mice exposed to LPS). Remarkably, the TNFR1/R2−/− (n = 12) and IL-1R−/− (n = 12) animals exposed to LPS and hyperthermia had a 0% mortality and the lpr mice had a 16.7% mortality (n = 2/12, all groups P < 0.0001 compared with C57BL/6 mice exposed to LPS and hyperthermia). Thus hyperthermia enhanced mortality in C57BL/6 mice treated with intratracheal LPS but did not increase mortality in TNFR1/R2−/−, lpr, or IL-1R−/− mice compared with C57BL/6 mice treated with LPS and maintained in euthermic conditions.

Febrile-range hyperthermia enhances LPS-induced lung injury by a mechanism requiring activity of the death receptors TNF and Fas. Bronchoalveolar lavage fluid (BALF) of mice exposed to intratracheal LPS (50 μg) and hyperthermia were assayed for total protein and IgM to assess changes in alveolar permeability (Fig. 3, A and B). At 48 h, our dose of LPS resulted in a modest increase in IgM in C57BL/6 mice exposed to euthermia (first column, Fig. 3A) (C57BL/6 mice exposed to PBS and euthermia BALF IgM 39.56 ng/ml ± 5.4 vs. C57BL/6 exposed to LPS and euthermia BALF IgM 253 ng/ml ± 13). Hyperthermia alone did not affect alveolar permeability (C57BL/6 exposed to intratracheal PBS and hyperthermia BALF IgM 59.24 ng/ml ± 7.5). However, when the C57BL/6 mice were exposed to hyperthermia, the administration of LPS resulted in significantly higher IgM concentrations (Fig. 3A). The TNFR1/R2−/− mice failed to show an enhancement in IgM BAL concentrations following hyperthermia, and a similar response was seen in the lpr mice. In contrast, the IL-1R−/− mice showed a response to hyperthermia that was similar to that of the C57BL/6 mice, with an increase in IgM concentrations compared with euthermic IL-1R−/− mice. A similar pattern of response was seen with the BAL protein content (Fig. 3B). These data suggest that death receptor pathways mediate the increased alveolar permeability associated with hyperthermia and LPS-induced lung injury.

In contrast, deficiency in IL-1 receptor activity does not prevent hyperthermia-augmented lung injury. For comparison, additional animals (n = 8) were exposed to hyperthermia (35°C to induce core body temperature of 39.5–40°C for 12 h, then 6 h recovery at 23°C) before the experimental protocol to induce heat shock because the heat shock response has been reported to be protective against ALI and mortality in animal models of direct and indirect lung injury (67, 72, 73). As in IL-1R−/− animals, the heat-treated animals were protected from augmented mortality associated with fever and intratracheal LPS (12.5% mortality in heat-treated WT mice exposed to fever + LPS vs. 70% mortality without heat pretreatment, P < 0.0001). Also similar to IL-1R−/− animals, the heat-treated animals were not protected from augmented alveolar permeability (BAL IgM (ng/ml) in heat-pretreated
animals exposed to LPS alone 204.1 ± 83.8 vs. heat-pretreated animals exposed to LPS and fever 899.8 ± 126, P < 0.01].

Lung inflammatory responses were measured using total cell and neutrophil (PMN) concentrations in BAL fluid and myeloperoxidase (MPO) activity in lung homogenates (Fig. 3, C–E). In contrast to the effect on permeability markers, exposure to hyperthermia did not enhance neutrophilic response to LPS at the 48-h point, and this was true for C57BL/6, TNFR1/R2−/−, and IL-1R−/− animals. Surprisingly, exposure to hyperthermia enhanced the BAL neutrophilic response of lpr animals to LPS.

A similar pattern of responses was seen for the lung total MPO activity, which reflects the total content of neutrophils in the lung, although the increase in lung MPO activity in lpr animals exposed to hyperthermia did not reach statistical significance. The MPO activity in TNFR1/R2−/− mice exposed to LPS and hyperthermia tended to be lower than wild-type mice, but this difference did not reach statistical significance. These data suggest that the augmented alveolar permeability associated with hyperthermia is not mediated directly by the pulmonary inflammatory response. The data show that deficiency in the Fas receptor increases total cell and PMN recovery in BAL fluid and that this increase in recovered inflammatory cells is not associated with increased alveolar permeability.

Lung tissue of animals exposed to LPS and euthermia or hyperthermia was examined for histological evidence of lung injury using light microscopy and for apoptosis using the TUNEL method (Fig. 4). C57BL/6 animals exposed to LPS and hyperthermia (Fig. 4, D–F) had increased alveolar wall thickness, microvascular congestion, and more TUNEL-positive cells compared with C57BL/6 animals exposed to LPS and euthermia (Fig. 4, A–C) (44). In contrast, under euthermic conditions the TNFR1/R2−/− mice showed few observable inflammatory cells and no evidence of alveolar thickening and displayed no enhancement with hyperthermia (Fig. 4, G and H). The lungs of TNFR1/R2−/− animals exposed to LPS and hyperthermia had fewer TUNEL-positive cells compared with C57BL/6 animals exposed to LPS and hyperthermia (Fig. 4 I). The lpr mice exposed to hyperthermia showed an increase in the numbers of PMN in the airspaces but only modest thickening of the alveolar septa compared with euthermic lpr mice (Fig. 4, J and K). The lpr mice exposed to LPS and hyperthermia also showed fewer TUNEL-positive cells compared with C57BL/6 mice (Fig. 4L). Animals deficient in IL-1R activity exposed to LPS and hyperthermia showed inflammatory cell accumulation, alveolar septal thickening, and TUNEL-positive cells similar to C57BL/6 mice exposed to LPS and hyperthermia (Fig. 4, M–O). Lung injury scores from each group (n = 2/group) exposed to intratracheal LPS for 48 h are presented in Table 2. The histological scoring system resulted in lung injury scores that were significantly correlated with BAL IgM con-
concentrations across all animal groups \( r = 0.835, P < 0.01 \), supporting the validity of this scoring approach. Animals lacking TNFR1/R2 activity had the lowest lung injury scores, which suggests that BAL analysis underestimated the protection from hyperthermia-augmented lung injury afforded by TNFR1/R2 deficiency.

To measure differences in the apoptotic signal, TUNEL-positive cells were counted in ten randomly generated \( \times 100 \) magnification fields of lungs of the four groups of animals exposed to LPS and hyperthermia for 48 h (Fig. 5). Compared with C57BL/6 mice exposed to LPS under euthermic conditions, the C57BL/6 mice exposed to LPS and hyperthermia had significantly more TUNEL-positive cells (44). However, the number of TUNEL-positive cells in the hyperthermic TNFR1/R2 \(^{-/-} \) and \( lpr \) mice was similar to the euthermic C57BL/6 mice. TUNEL positivity was significantly higher in the IL-1R \(^{-/-} \) animals exposed to LPS and hyperthermia compared with the C57BL/6 euthermic mice. Thus hyperthermia increases the number of apoptotic cells in the lungs of WT animals exposed to LPS, and this response requires expression of TNFR and Fas.

**Febrile-range hyperthermia suppresses pulmonary TNF-\( \alpha \) and KC production after LPS-induced injury.** A multiplex assay of chemokines and cytokines in lung homogenates was performed in C57BL/6 mice exposed to intratracheal PBS or LPS and euthermia or hyperthermia for up to 48 h (Fig. 6). At 24 h, exposure to hyperthermia had no effect on the lung concentrations of all of the cytokines tested. However, at 48 h, the hyperthermic mice had significantly lower concentrations of TNF-\( \alpha \) and KC and higher concentrations of MIP-2. At 48 h, hyperthermic animals also had trends toward lower concentrations of IL-1\( \beta \) and GM-CSF and higher concentrations of IL-6 in lung homogenates than euthermic animals although these differences did not reach statistical significance. These data suggest that hyperthermia has discrete effects on the pulmonary inflammatory cytokine response after LPS-induced lung injury and decreases the concentration of several critical inflammatory mediators in the lungs of animals with fever and lung injury.

**Febrile-range hyperthermia during LPS-induced lung injury is associated with evidence of systemic inflammation.** Concentrations of nitric oxide (NO) metabolites and malondialdehyde (MDA), a marker of lipid peroxidation, were measured in the plasma of wild-type and genetically abnormal mice exposed to intratracheal LPS and euthermia or hyperthermia. The wild-type animals exposed to LPS and hyperthermia had significantly higher plasma concentrations of NO metabolites and MDA compared with wild-type animals exposed to LPS and euthermia at both 24 and 48 h (Fig. 7). Animals lacking TNFR1/R2, Fas or IL-1 receptor activity exposed to LPS and hyperthermia had plasma concentrations of NO metabolites and MDA that were significantly lower than wild-type animals exposed to hyperthermia and LPS at both 24 and 48 h. The plasma markers of systemic injury correlated with the mortality rates in these animals. These findings were similar in the heat-pretreated animals. Thus deficiencies in activity of the death receptors TNFR1/R2 or Fas, or IL-1 receptor activity, are associated with attenuated systemic oxidative and nitrosative stress after exposure of mice to intratracheal LPS and hyperthermia.

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Fig. 3. A deficiency in the activity of death receptors TNFR1/R2 or Fas but not in IL-1 receptor activity attenuates hyperthermia-augmented lung injury. IgM (A) and total protein (B) concentrations in bronchoalveolar lavage (BAL) fluid collected at 48 h are increased in WT or IL-1R \(^{-/-} \) animals exposed to LPS and hyperthermia compared with WT or IL-1R \(^{-/-} \) animals exposed to LPS and euthermia or hyperthermia alone (data not shown). Hyperthermia does not affect IgM and total protein concentrations in TNFR1/R2 \(^{-/-} \) or \( lpr \) animals exposed to LPS. Total cell (C) and polymorphonuclear neutrophils (PMN) (D) concentrations in BAL fluid collected at 48 h were similar in all groups exposed to LPS and euthermia or hyperthermia with the exception of \( lpr \) mice, which had increased concentrations of cells and PMN in BAL fluid. Lung homogenerate myeloperoxidase (MPO) activity (E) was unaffected by the presence of hyperthermia in all groups exposed to LPS for 48 h. \( * P < 0.05 \), \( § P < 0.001 \). Animal numbers in each experimental group are noted in Table 1.

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**L64 EFFECT OF FEVER ON LUNG INJURY**

AJP-Lung Cell Mol Physiol • VOL 301 • JULY 2011 • www.ajplung.org

![Graphs and images](http://ajplung.physiology.org/ by 10.220.32.247 on November 6, 2017)
DISCUSSION

We previously demonstrated that fever enhances lung injury and mortality in mice exposed to intratracheal LPS. This enhancement was associated with increased alveolar epithelial apoptosis in vivo and with increased TNF-α-mediated apoptosis associated with NF-κB suppression and decreased inflammatory cytokine production in vitro (44). Interestingly, we found that the effects of hyperthermia in vitro were specific to the TNF-α receptor and that hyperthermia had no effect on cell death mediated by Fas.

Therefore, we hypothesized that fever augments lung injury and mortality through the TNF-α receptor in vivo. We tested this hypothesis by exposing animals lacking activity of one of two of the major representatives of the death receptor family, TNFR1/R2 or Fas, to intratracheal LPS and hyperthermia. As a comparison for cell death and inflammation mediated by pathways other than death receptors and to evaluate the role of a classical pyrogen in hyperthermia-augmented lung injury, we also treated mice lacking IL-1 receptor activity to intratracheal LPS and euthermia or hyperthermia. We anticipated that animals lacking TNF-α receptor activity would be resistant to the effects of hyperthermia on lung injury and mortality and that those animals lacking Fas or IL-1 receptor activity would be more susceptible to augmented lung injury.

Fig. 4. A deficiency in the activity of the death receptors TNFR1/R2 or Fas, but not IL-1 receptor deficiency, protects from hyperthermia-augmented lung injury and apoptosis. Hematoxylin and eosin stains of paraffin-embedded lungs of wild-type (D–F) and IL-1R−/− animals (M–O) exposed to LPS and hyperthermia for 48 h show increased alveolar septal thickening and TUNEL-positive cells (indicated by arrows) compared with wild-type animals exposed to LPS and euthermia (A–C) or TNFR1/R2−/− (G–I) or lpr (J–L) animals exposed to LPS and hyperthermia. The histological examination of lung tissue sections of wild-type, lpr, and IL-1R−/− animals show similar PMN tissue recruitment, whereas TNFR1/R2−/− animals show significantly fewer PMNs.
and mortality associated with fever. However, we found that mice lacking activity of either of the two members of the death receptor family evaluated were protected from lung injury and mortality augmented by hyperthermia. Interestingly, we found that mice lacking IL-1 receptor activity were not protected from enhanced lung injury associated with fever but were protected from associated mortality. Similarly, heat-pretreated animals were not protected from enhanced lung injury associated with fever but were protected from associated mortality and changes in plasma concentrations of MDA and NO metabolites. We also found that fever decreases the concentration of several important inflammatory cytokines in the lungs after LPS exposure although fever increases systemic evidence of inflammatory injury, specifically oxidative and nitrosative stress. Furthermore, we found that plasma markers of inflammatory injury after exposure to LPS and hyperthermia were associated with mortality. These data show for the first time that hyperthermia enhances lung injury in vivo by a mechanism involving death receptor signaling. However, the data also show that the mortality seen in hyperthermic mice is not directly associated with the severity of lung injury and may relate to a systemic inflammatory response.

Ligands of the death receptor family, including TNF-α, Fas ligand and TRAIL, are present in the lungs of patients with ALI and have been implicated in both inflammation and apoptosis in the pathogenesis of experimental ALI. Previous studies demonstrated that stimulation of the Fas receptor in the alveolar epithelium causes alveolar epithelial apoptosis and causes ALI in an experimental model and that Fas ligand concentrations are elevated in humans with fatal adult respiratory distress syndrome (ARDS) compared with survivors (19, 49–52). Bem et al. (5) demonstrated that TRAIL is elevated in the BAL fluid of children with severe respiratory syncytial virus infection compared with ventilated children without pulmonary disease and that TRAIL is important in death of airway epithelial cells. Park, Parsons, and other groups have demonstrated that soluble concentrations of TNFR1, TNFR2, and TNF-α are found in BAL fluid and plasma in patients with ARDS and may correlate with severity of disease (31, 60–62). Therefore, death receptors are clinically important mediators of ALI.

The present data support the in vivo relevance of our in vitro observations about the TNF receptor pathway in lung injury augmented by fever (44). We found that fever increased the number of TUNEL-positive cells in the lungs of C57BL/6 mice exposed to inhaled LPS. We hypothesized that fever specifically enhanced lung injury through the TNF-α receptor on the basis of our previous in vitro work, and we found on histopathological examination that animals lacking TNFR1/R2 activity were more protected from hyperthermia-enhanced injury than animals lacking Fas or IL-1R activity. However, we found that animals deficient in either of the death receptor family representatives, TNF-α or Fas receptor, exposed to LPS and hyperthermia had attenuated alveolar permeability and fewer TUNEL-positive cells compared with C57BL/6 animals exposed to LPS and hyperthermia. These data suggest that the enhanced lung injury associated with fever requires death receptor activity. However, unlike the in vitro observations, the effect of fever on lung injury in vivo is not specific to TNF-α but rather seems to reflect an effect of temperature on the family of death receptors, which are homologous at multiple sites. The discrepancy between the in vivo results reported here and the previously reported in vitro findings may relate to differences in the presentation of the death receptor ligands to death receptors in vitro and in vivo. Therefore, we speculate that temperature affects death receptor activity at a site common to multiple members of the death receptor family.

Others have observed that febrile-range hyperthermia enhances experimental lung injury (1, 3, 29, 67, 69). Ball et al. (3) and Hasday et al. (29) demonstrated that hyperthermia enhances the pulmonary inflammatory response after injurious ventilation in preterm lambs or during pulmonary oxygen toxicity in CD-1 mice, respectively. However, our data reproducibly demonstrate that hyperthermia can augment alveolar permeability through mechanisms other than inflammation, which may reflect variable responses generated in separate

### Table 2. Lung injury scores

<table>
<thead>
<tr>
<th>Temperature</th>
<th>C57BL/6</th>
<th>TNFR1/R2−/−</th>
<th>lpr</th>
<th>IL-1R−/−</th>
</tr>
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<tbody>
<tr>
<td>Euthermia</td>
<td>2.35 ± 0.35</td>
<td>2.40 ± 0.07</td>
<td>2.38 ± 0.12</td>
<td>2.28 ± 0.55</td>
</tr>
<tr>
<td>Hyperthermia</td>
<td>3.65 ± 0.25</td>
<td>1.87 ± 0.13</td>
<td>2.50 ± 0.10</td>
<td>2.83 ± 0.07</td>
</tr>
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Values are means ± SE. Lung injury score was calculated on animals exposed to LPS (50 μg), intratracheal, for 48 h.
species or strains of mice (3, 29, 67). We did not find significant alteration in LPS-induced lung inflammation in animals lacking TNFR1/R2 compared with C57BL/6 mice, which was unexpected on the basis of available literature suggesting that TNFR1 facilitates BAL PMN recruitment but also may attenuate tissue inflammation after LPS (9, 68). We found that Fas-abnormal (lpr) animals exposed to LPS and hyperthermia have increased BAL PMN concentrations compared with all other groups although total lung PMNs, inferred from lung homogenate MPO activity, were unchanged by hyperthermia in lpr mice exposed to LPS. Despite the increase in recovered neutrophils from BAL, lpr mice are protected from enhanced alveolar permeability associated with fever compared with C57BL/6 mice. The increases in BAL PMN concentration in the hyperthermic lpr animals and in MPO activity in both euthermic and hyperthermic lpr animals were unexpected, as previous work from our laboratory demonstrated that Fas-pathway activity is important to sustained lung inflammation, assessed by BAL PMN activity (53). The change in leukocyte recruitment may reflect differences in the experimental exposures between our present study and the previous report, including LPS dose and the presence of hyperthermia, as multiple studies suggest that the activity of the Fas pathway is highly affected by microenvironmental factors (18, 30, 75).

However, we did find a trend toward lower BAL concentrations of PMN in lpr mice treated with LPS alone. Therefore, hyperthermia may affect the recovery of BAL PMN in mice lacking Fas activity, but this is not addressed in the present study. Mice lacking IL-1R activity treated with LPS and euthermia showed a slight trend toward fewer cells and PMNs in BAL than C57BL/6 mice, and IL-1R−/− mice exposed to LPS and hyperthermia show a trend toward more BALF PMNs than C57BL/6 although neither trend reached statistical significance.

We found that several important inflammatory cytokines in the lungs of C57BL/6 mice exposed to LPS and hyperthermia were decreased compared with animals exposed to LPS and euthermia. We previously reported that hyperthermia suppresses NF-κB activity and cytokine transcription in alveolar epithelial cell lines, and others have observed a similar effect in monocyte-derived cells (14, 15). Interestingly, mice without IL-1 receptor activity, an important component of the inflammatory response, were susceptible to hyperthermia-enhanced lung injury. These
data also suggest that the mechanism by which fever enhances alveolar permeability associated with lung injury is not dependent on pulmonary inflammation in our model.

We observed that hyperthermia increases mortality after lung injury by a complex interaction between inflammation and death receptor activity. Akinci et al. (1) demonstrated that concomitant exposure of rats to hyperthermia and an injurious ventilator strategy for 1 h increased systemic inflammation, assessed by serum cytokine concentrations (TNF-α, monocyte chemottractant protein 1, IL-1β, IL-6), compared with normothermic or hypothermic animals exposed to low-pressure or high-pressure ventilation although no changes in mortality or evaluation of cytokine responses at later time points were noted in this brief experimental model (1). Others have also observed that hyperthermia modifies the systemic inflammatory response although this change in inflammation has not been associated with increased mortality (33–35). Although we found that hyperthermia suppresses pulmonary inflammatory cytokine concentrations after LPS exposure, we found that hyperthermia increases plasma markers of inflammatory stress, specifically NO metabolites and lipid peroxidation after LPS exposure in C57BL/6 mice. In contrast, animals without IL-1 or death receptor activity exposed to LPS and fever did not show evidence of inflammatory stress in the plasma. Therefore, plasma markers of oxidative and nitrosative stress correlate directly with mortality and do not correlate with the presence of lung injury enhanced by fever.

NO is a broadly active signaling molecule under the influence of inflammatory cytokines and the direct effect of endotoxin that directly modifies vascular tone and immune function and contributes to free radical stress during sepsis and the systemic inflammatory response (7, 13, 20, 45, 46). Although hyperthermia in the range of heat shock (43°C) can directly induce NO synthase and cause systemic hypotension and death in a rat model of heat stroke, our model of fever-range hyperthermia did not independently affect plasma concentration of NO metabolites (23, 41). This study does not address whether the increased NO concentration observed in the mice exposed to hyperthermia and LPS was attributable to direct induction of NO synthase or the effect of increased production of inflammatory cytokines in the peripheral blood leukocytes. However, the increase in NO metabolites correlates with mortality and may directly contribute to mortality in this model by causing maladaptive systemic vasodilatation or mitochondrial dysfunction.

Reactive oxygen species (ROS) are physiological end-products of normal oxygen metabolism. Increased production of ROS by mitochondrial dysfunction, tissue injury, and mobilized leukocytes can overwhelm innate ROS scavenger mechanisms during sepsis, for example, resulting in oxidative stress (27). Oxidative stress has been associated with both ALI and sepsis and correlates with poor outcomes in the critically ill (2, 24, 25). We observed evidence of enhanced lipid peroxidation, a marker of oxidant stress, in the plasma of mice exposed to LPS and febrile-range hyperthermia but not in the plasma of animals exposed to LPS alone or hyperthermia alone. This study did not address the source of the oxidative stress directly but provides evidence that febrile-range hyperthermia can convert lung injury into a systemic phenomenon that increases risk of mortality.

In conclusion, we show for the first time that TNF receptor and Fas signaling are required for hyperthermia-enhanced lung injury.
in vivo. We found that hyperthermia enhances lung injury without affecting inflammatory cell recruitment to the lung and is associated with diminished concentrations of several inflammatory cytokines in the lung. We found that hyperthermia enhances mortality by a mechanism associated with increases in markers of oxidative and nitrosative stress but not as a direct consequence of lung injury. Finally, we observed that fever converts a moderate, survivable model of ALI into a systemic inflammatory syndrome with high mortality. The data suggest that febrile-range temperatures might have an adverse effect in patients with ALI and that studies of the effect of fever on the outcome of patients with ALI are needed.

REFERENCES

EFFECT OF FEVER ON LUNG INJURY


