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Extracellular Histones Are Mediators of Death through TLR2 and TLR4 in Mouse Fatal Liver Injury

Jun Xu,* Xiaomei Zhang,† Marc Monestier,‡,§ Naomi L. Esmon,* and Charles T. Esmon*,†,¶,‖

We previously reported that extracellular histones are major mediators of death in sepsis. Infusion of extracellular histones leads to increased cytokine levels. Histones activate TLR2 and TLR4 in a process that is enhanced by binding to DNA. Activation of TLR4 is responsible for the histone-dependent increase in cytokine levels. To study the impact of histone release on pathology we used two models: a Con A-triggered activation of T cells to mimic sterile inflammation, and acetaminophen to model drug-induced tissue toxicity. Histones were released in both models and anti-histone Abs were protective. TLR2- or TLR4-null mice were also protected. These studies imply that histone release contributes to death in inflammatory injury and in chemical-induced cellular injury, both of which are mediated in part through the TLRs. ***The Journal of Immunology*****, 2011, 187: 2626–2631.**

Systemic inflammatory responses syndrome (SIRS) occurs in a variety of medical conditions, including infection, trauma, ischemia-reperfusion, as well as autoimmunity. Sepsis is a typical example of SIRS caused by infection. Both Gram-negative and -positive bacteria can cause SIRS through TLR signaling pathways (1). The clinical sequelae of SIRS include organ injury, increases in coagulation that may result in macro- and microvascular thrombosis, and tissue injury mediated by cytokines and leukocytes (1, 2). In sepsis and trauma, there are elevated levels of circulating nucleosomes that could be derived either from apoptotic cells (3) or from the degradation of neutrophil extracellular traps (4, 5). Apoptosis and tissue necrosis increase in severe diseases such as sepsis and trauma and correlate with mortality (3, 6, 7). Nucleosomes result from chromatin degradation and consist of a core octamer of two copies of histones H2A, H2B, H3, and H4 wrapped by a 146 bp of DNA. Recently, neutrophil extracellular traps were shown to activate platelets leading to thrombosis (5), and the major contributor to this process was histone H4. We previously reported that extracellular histones released in response to bacterial challenge are mediators contributing to endothelial dysfunction, organ failure, and death during sepsis. In that study, histone H4 was also shown to kill endothelial cells (8). Most importantly, blocking histone-mediated cytotoxicity was shown to protect mice from LPS-mediated death. Infusion of histones alone into mice resulted in a pathological response similar to sepsis, including migration of leukocytes into tissues, microvascular thrombosis, and organ failure, ultimately culminating in death.

The molecular mechanisms of histone-mediated tissue injury and organ failure remain unclear. In this study, we demonstrate that TLR2 and TLR4 are major receptors for extracellular histone-mediated sterile inflammation, tissue injury, and death in mouse models. Extracellular histones and their signaling pathways through TLRs are potential novel therapeutic targets in a variety of inflammatory and chemical toxin-mediated diseases.

Materials and Methods

Reagents

We purchased Con A (C2010), calf thymus histones (H9250), calf thymus DNA (D1501), and acetaminophen (APAP) (A5000) from Sigma-Aldrich and goat Ab to H3 from Santa Cruz Biotechnology. Dr. Marc Monestier provided mouse mAbs to H3 (LG2-1), H4 (BWA-3), and DNA–H2A–H2B (PR1-3).

Animals

We used 6- to 12-wk-old male wild-type (WT) C57BL/6, TLR2 knockout (KO) (stock no. 004650, backcrossed at least seven generations), and TLR4 KO (stock no. 007227, backcrossed at least six generations) mice (The Jackson Laboratory) according to an animal protocol approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

Mouse plasma chemistry, cytokine assay, and Western blotting

We collected whole mouse blood in 11 mM sodium citrate by heart puncture and isolated plasma by centrifuging the blood immediately after collection. We added 20 mM benzamidine to the citrated blood to isolate the plasma for Western blotting of H3 with goat Ab to H3. We measured mouse plasma alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatine kinase, creatinine, and total bilirubin with a Vitros 250 chemistry analyzer (Ortho Clinical Diagnostics) and IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, KC, and TNF-α with an MSD 96-well multi-array for mouse cytokines (Meso Scale Diagnostics). The original histone TLR screening assay was performed by InvivoGen.

Cell culture and TLR signaling assay

We cultured human TLR2 (HEK-Blue-2)- or human TLR4 (HEK-Blue-4)-expressing HEK293 cells in DMEM supplemented with 10% FBS and selective antibiotics according to the manufacturer's instructions (InvivoGen). The TLR4-expressing cells also express CD14. We measured secreted alkaline phosphatase as a reporter gene under the control of a promoter inducible by NF-κB to monitor the signaling by histones through TLR2 or TLR4 using HEK-Blue detection medium according to

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The online version of this article contains supplemental material.

Abbreviations used in this article: APAP, acetaminophen; DAMP, damage-associated molecular pattern; KO, knockout; SIRS, systemic inflammatory responses syndrome; WT, wild-type.

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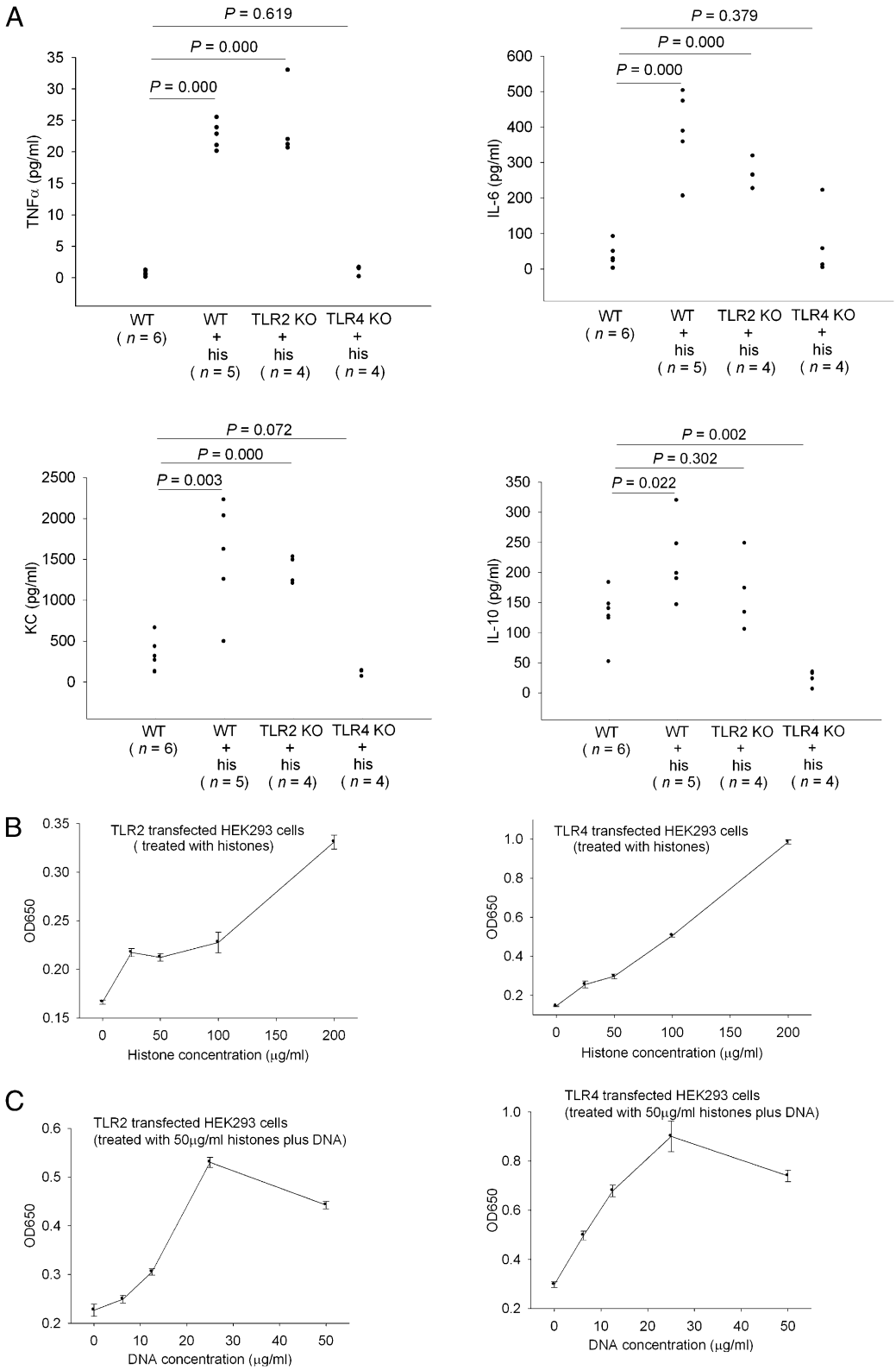

FIGURE 1. Extracellular histones trigger TLR2 and TLR4 signaling. *A*, TNF-α, IL-6, KC, and IL-10 levels in WT, TLR2 KO, or TLR4 KO mouse plasma collected 2 h after an i.v. injection of calf thymus histones (25 mg/kg body weight). Data are from five experiments. *B*, Reporter gene expression levels in TLR2- or TLR4-transfected HEK293 cells stimulated with calf thymus histones at the indicated concentrations. The results shown are the means ± SD of experiments performed in triplicate. *C*, Reporter gene expression levels in TLR2- or TLR4-transfected HEK293 cells stimulated with calf thymus histones (50 μg/ml) and calf thymus DNA at the indicated concentrations. The results shown are the means ± SD of experiments performed in triplicate.

the manufacturer's instructions (InvivoGen). In experiments to determine the effect of DNA on histone signaling in vitro, the DNA was sonicated into 100- to 1000-bp fragments, mixed with the histones, and immediately added to the cells.

Statistical analysis

We expressed the results as means ± SD and used a Student *t* test to compare two groups. We analyzed survival studies with the log-rank test in the program

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Prism (GraphPad Software). We considered differences statistically significant at a p value of <0.05.

Results

Extracellular histones trigger TLR2 and TLR4 signaling

Extracellular histones released in response to inflammatory challenge contribute to endothelial dysfunction, organ failure, and death during sepsis (8). We wanted to further explore the molecular mechanisms of histone-mediated tissue injury and lethality. To avoid the direct histone toxicity toward cells, a sublethal dose (25 mg/kg) of histones was injected into WT (C57BL/6) mice. We found high levels of TNF-α, IL-6, KC, and IL-10 in the circulation of WT mice 2 h after histone injection (Fig. 1*A*). Because TLRs are obvious candidates for signaling inflammatory pathways, we screened seven TLRs as possible histone signaling receptors. TLR2 and TLR4, but not TLR3, TLR5, TLR7, TLR8, or TLR9, were stimulated by histones in TLR-transfected cell lines. The presence of DNA further increased the histone-induced TLR2 and TLR4 signaling (Fig. 1*B*, 1*C* and data not shown). Boiling the histones and DNA abolished TLR2 and TLR4 signaling, ruling out possible LPS effects in these experiments (data not shown). The massive proinflammatory cytokine production induced by histones was abolished in TLR4 KO mice but not in TLR2 KO mice (Fig. 1*A*), suggesting that TLR4 is the major receptor for a histone-induced cytokine inflammatory response.

Extracellular histones are major mediators of death through TLR2 and TLR4 in a Con A-induced sterile inflammation model

In our previous report, we demonstrated that extracellular histones released in response to bacterial inflammatory challenges contributed to endothelial dysfunction, organ failure, and death during sepsis (8). To determine whether the same is true for a sterile inflammatory challenge, a Con A challenge was used to induce a T cell activation-dependent inflammatory reaction (9). A lethal dose of Con A (30 mg/kg) was injected i.v. and 40 min later we injected mAb to histone H3 or mouse isotype control Ab. Among the effects of Con A infusion, liver injury is caused by the infiltration of activated T cells, which results in massive necrotic tissue injury and inflammation (9). We hypothesized that tissue necrosis in this model might result in histone release, contributing to inflammation and death. Indeed, we detected intact H3 in the plasma after Con A challenge by Western blotting (Fig. 2*A*), which was sustained for at least 4 h. Injection of H3 Ab rescued seven of eight mice (Fig. 2*B*), indicating that extracellular histones are major mediators of death in this sterile inflammation model, although the H3 Ab appeared to have no influence on the level of histones present in plasma after Con A challenge (Fig. 2*A*). H3 Ab had no significant protective effect on liver injury caused by Con A since H3 Ab injection did not decrease the high levels of alanine aminotransferase or aspartate aminotransferase in mouse plasma either 2 or 6 h after Con A challenge (Supplemental Fig. 1). This result is consistent with a previous finding that liver damage itself was not a major

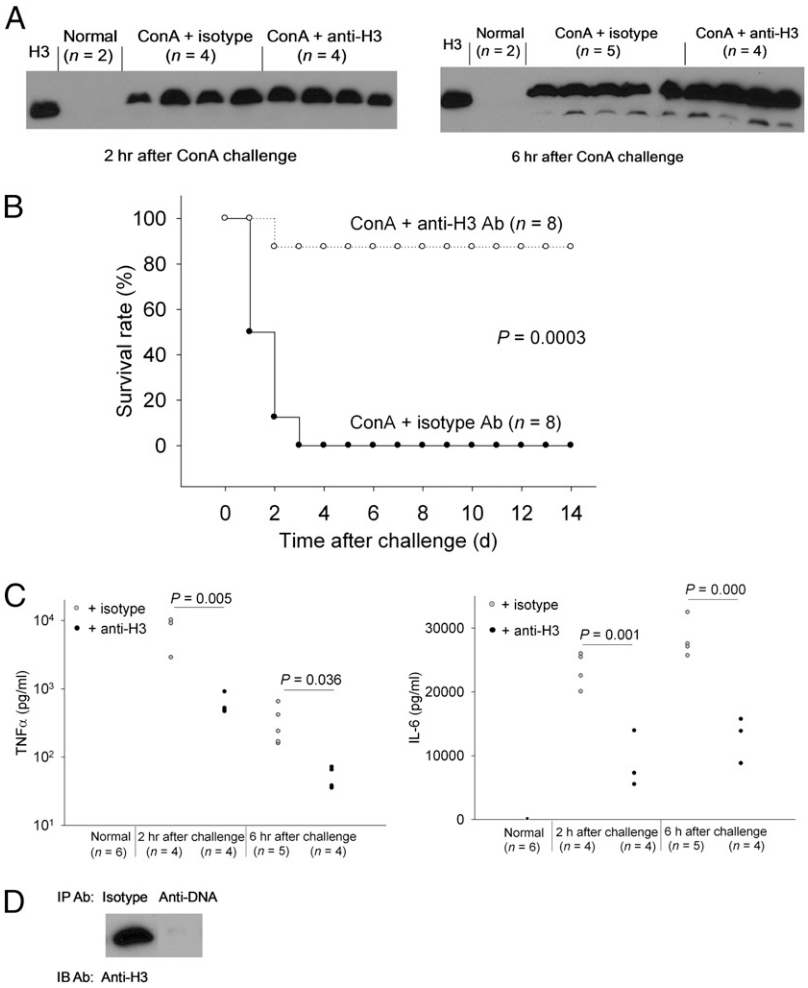

FIGURE 2. Extracellular histones are major mediators of death in a Con A-induced sterile inflammation model. *A*, Western blot analysis using a goat polyclonal anti-H3 in individual mouse plasmas collected 2 or 6 h after lethal Con A (30 mg/kg body weight) challenge in the presence of mouse mAb to H3 or mouse isotype control Ab (10 mg/kg body weight). The *left lane* is the H3-positive control. *B*, Survival rates of C57BL/6 mice challenged with a lethal dose of Con A (30 mg/kg body weight, i.v.) and 40 min later treated with Ab to H3 or mouse isotype control Ab (10 mg/kg body weight, i.v.). Data are from three experiments. *C*, Plasma TNF-α and IL-6 levels in mice without challenge or in Con A-challenged mice as in *A*. Data are from three experiments. *D*, Western blot analysis for H3 in a Con A-challenged mouse plasma sample after immunoprecipitation by either isotype control or DNA Ab.

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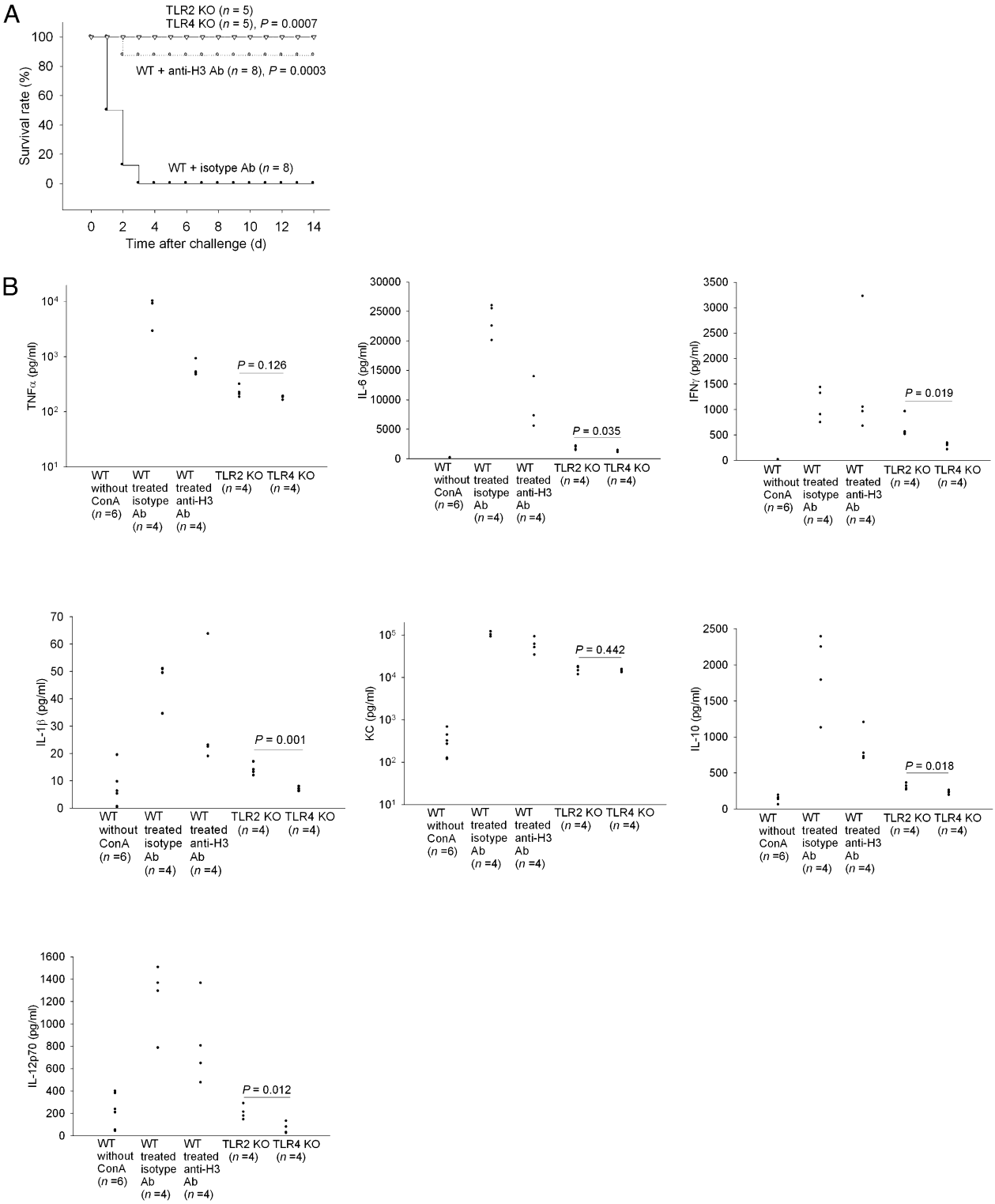

FIGURE 3. TLR2 KO or TLR4 KO mice are resistant to a lethal challenge of Con A. *A*, Survival rates of TLR2 KO or TLR4 KO mice injected i.v. with a lethal dose of Con A (30 mg/kg body weight), combined with the data for WT mice shown in Fig. 2*B*. The *p* values are as compared with WT mice treated with isotype control Ab. Data are from five experiments. *B*, TNFα, IL-6, IFN-γ, IL-1β, KC, IL-10, and IL-12p70 levels in TLR2 KO, TLR4 KO, or WT mouse plasma collected 2 h after the lethal challenge of Con A. Data for TNF and IL-6 from untreated WT mice are repeated from Fig. 2*C*. Data are from five experiments.

contributor to death in the Con A model (10). The H3 Ab also did not decrease the high levels of creatine kinase or total bilirubin in the circulation after Con A challenge (Supplemental Fig. 2). However, we found that H3 Ab did significantly reduce TNF-α and IL-6 levels in the circulation of the Con A-challenged mice (Fig. 2*C*). The observed reduction in cytokine levels by Ab against H3 in this sterile inflammation model is consistent with extracellular histones being able to signal TLRs for cytokine production. To test whether the extracellular histones in the circulation of Con A-challenged mice were still complexed with DNA, we immu-

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noprecipitated DNA in a plasma sample with Ab against DNA–H2A–H2B complex and measured the H3 level remaining in the supernatant by Western blotting. We found that the DNA–H2A–H2B Ab removed most of the H3 in the sample compared with the isotype control Ab (Fig. 2*D*), indicating that the bulk of the extracellular histones detected by Western blotting were still complexed with DNA in the circulation in this mouse model.

To test whether TLR2 and/or TLR4 contributed to the Con A-induced lethality in this fatal inflammation model, we injected a lethal dose of Con A into TLR2 KO and TLR4 KO mice and found that both were protected from the challenge (Fig. 3*A*). Even though liver injury still occurred in the KO mice 2 h after Con A challenge (Supplemental Fig. 3), circulating levels of TNF-α, IL-6, IFN-γ, IL-1β, KC, IL-10, and IL-12p70 levels were lower than those observed in H3 Ab-treated WT mice (Fig. 3*B*). We also found that IL-6, IFN-γ, IL-1β, IL-10, and IL-12p70 levels were significantly lower in the circulation of Con A-challenged TLR4 KO mice than in the TLR2 KO mice (Fig. 3*B*), consistent with our previous finding that TLR4 might be a major receptor for histone-induced cytokine production.

Extracellular histones are major mediators of death through TLR2 and TLR4 in an APAP-induced tissue injury model

Having established an important role for histone-mediated lethality that involves TLR2 and TLR4 in both pathogen and sterile inflammatory challenges, we wanted to determine whether cell injury in the absence of a massive inflammatory reaction also involved histones and TLR signaling. APAP is known to induce liver injury and lethality in mice mimicking drug-induced liver injury in patients with APAP overdose (11). HMGB1 and DNA released from dying hepatocytes as damage-associated molecular pattern (DAMP) molecules were reported to be responsible for the activation of innate immunity and tissue damage following APAP hepatotoxicity in this mouse model (11, 12). We hypothesized that extracellular histones as unrecognized endogenous DAMP molecules are also important mediators in APAP-induced liver injury and lethality through TLR2 and/or TLR4 signaling. Although we found extracellular histone in the circulation after APAP challenge (Fig. 4*A*), we did not observe detectable TNF-α and IL-1β in the circulation (data not shown). Abs to histones H3 or H4 protected the WT mice from APAP-induced lethality, and both TLR2 and TLR4 KO mice were also resistant to the APAP challenge (Fig. 4*B*). These results are consistent with the previous finding that TLR4 mutant mice were less susceptible to liver injury after a sublethal APAP challenge and the notions that endogenous DAMP molecules augment inflammation and/or tissue injury through TLRs and other signaling molecules (13, 14).

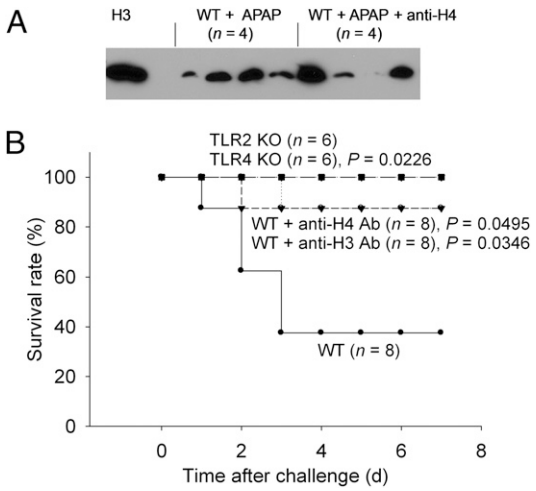

FIGURE 4. TLR2 KO or TLR4 KO mice are resistant to a lethal challenge of APAP. *A*, Western blot analysis for H3 in individual WT mouse plasmas collected 6 h after a high dose of APAP (500 mg/kg body weight, i.p.) in the absence or presence of Ab to H4 (10 mg/kg body weight, iv). The *left lane* is the H3-positive control. *B*, Survival rates of WT and TLR2 KO or TLR4 KO mice challenged with a high dose of APAP (500 mg/kg body weight, i.p.) and WT mice in the absence or presence of Ab to H3 or H4 (10 mg/kg body weight, i.v.). A *p* value is compared with WT mice challenged with APAP. Data are from five experiments.

Discussion

Superficially, there appears to be a conflict in the data presented in this study. We show that histones induce the production of circulating cytokines. We also show that APAP leads to the appearance of a significant concentration of histones in the circulation. However, we do not detect concomitant cytokines. This could be due to retention of the proteins in the liver without release into the bloodstream or intracellular destruction. Consistent with this observation, Imaeda et al. (11) found that although the messages for pro–IL-1β, IL-18, TNF-α, and IFN-γ were increased by APAP 2-fold or more in a similar model, only a 5% increase in IL-1β over baseline was observed in the circulation.

The cytokine storm is a hallmark of the systemic host inflammatory response to infection as well as injury. LPS-induced TLR4 signaling is a well-known pathway responsible for cytokine production in infectious diseases, including sepsis (15). Endogenous TLR4 ligands such as heat shock proteins, fibrinogen, HMGB1, and tenascin-C have been reported, but only HMGB1 and tenascin-C were able to induce proinflammatory cytokine synthesis in vitro and in vivo (15–17). We now show that extracellular histones activate TLR4 signaling in vitro and induce cytokine production in vivo primarily through TLR4 activation. Extracellular histones, DNA, or both were found in the circulation of the Con A-induced sterile inflammatory mouse model in this study and in patients of stroke, trauma, and autoimmune diseases (18). However, TLR2 KO mice were resistant to the lethal challenges of Con A, suggesting that histone-mediated TLR2 signaling might be responsible for tissue dysfunction at late stages after the cytokine storm.

The ability of histones to activate TLR2 and TLR4 is indicated by the reporter gene experiments in the transfected HEK293 cells. In vivo, the TLR2-null mice were protected to the same extent from APAP toxicity as was achieved by blocking histone H4. The simplest interpretation of this result is that histones contribute to toxicity by activating TLR2, but we cannot exclude the possibility that other mediators might be released by the histones that activate TLR2 and contribute to death. Similar considerations apply to the interpretation of the TLR4-null mice and the impact of histone blockade in Con A-mediated injury. Recent studies demonstrated that TLR2 KO mice were more resistant to atherosclerosis, ischemic tissue injury, type 1 diabetes, and mouse lupus (19–22), suggesting the presence of endogenous TLR2 agonists, which might contribute to the pathogenesis of these diseases. Extracellular histones are good candidates for this endogenous ligand. The exact molecular mechanisms of histone-mediated TLR2 signaling are under investigation.

A massive inflammatory response often follows septic tissue injury or trauma (23). Previously, the role of activated macrophages recruited to the site of injury has been considered a major culprit (24). Consistent with our observations on the effect of histones, especially when complexed with DNA, nucleosomes (our unpublished results) or DNA is readily detected in trauma patients and correlates with the severity of the disease (25, 26). Our studies demonstrating that DNA–histone complexes, as seen

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in Fig. 2*D*, can activate TLR2 and TLR4, leading to cytokine production and tissue injury, also complement another recent study showing that mitochondrial formyl peptides and mitochondrial DNA can activate TLR9 and lead to activation of human polymorphonuclear neutrophils (27). Thus, cellular damage elicits at least two distinct mechanisms that trigger inflammation and can contribute to the association of inflammation with trauma.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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