NLRP3 Inflammasome Deficiency Protects against Microbial Sepsis via Increased Lipoxin B₄ Synthesis

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Abstract

Rationale: Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection, is a major public health concern with high mortality and morbidity. Although inflammatory responses triggered by infection are crucial for host defense against invading microbes, the excessive inflammation often causes tissue damage leading to organ dysfunction. Resolution of inflammation, an active immune process mediated by endogenous lipid mediators (LMs), is important to maintain host homeostasis.

Objectives: We sought to determine the role of the nucleotide-binding domain, leucine-rich repeat-containing receptor, pyrin domain-containing-3 (NLRP3) inflammasome in polymicrobial sepsis and regulation of LM biosynthesis.

Methods: We performed cecal ligation and puncture (CLP) using mice lacking NLRP3 inflammasome-associated molecules to assess mortality. Inflammation was evaluated by using biologic fluids including plasma, bronchoalveolar, and peritoneal lavage fluid. Local acting LMs in

peritoneal lavage fluid from polymicrobial septic mice were assessed by mass spectrometry-based metabololipidomics.

Measurements and Main Results: Genetic deficiency of NLRP3 inhibited inflammatory responses and enhanced survival of CLP-induced septic mice. NLRP3 deficiency reduced proinflammatory LMs and increased proresolving LM, lipoxin B₄ (LXB₄) in septic mice, and in macrophages stimulated with LPS and ATP. Activation of the NLRP3 inflammasome induced caspase-7 cleavage and pyroptosis. Caspase-7 deficiency similarly reduced inflammation and mortality in CLP-induced sepsis, and increased LXB₄ production *in vivo* and *in vitro*. Exogenous application of LXB₄ reduced inflammation, pyroptosis, and mortality of mice after CLP.

Conclusions: Genetic deficiency of NLRP3 promoted resolution of inflammation in polymicrobial sepsis by relieving caspase-7-dependent repression of LXB₄ biosynthesis, and increased survival potentially via LXB₄ production and inhibition of proinflammatory cytokines.

Keywords: lipid mediators; NLRP3 inflammasome; sepsis

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At a Glance Commentary

Scientific Knowledge on the

Subject: Despite recent developments in clinical management and intervention, sepsis remains one of the leading causes of mortality and critical illness worldwide. Although excess inflammatory responses during sepsis can often cause severe tissue damage and organ dysfunction leading to death, the specific role of the nucleotide-binding domain, leucine-rich repeat-containing receptor, pyrin domain-containing-3 inflammasome-mediated immune response remains unknown.

What This Study Adds to the

Field: This study suggests a critical role for the nucleotide-binding domain, leucine-rich repeat-containing receptor, pyrin domain-containing-3 inflammasome in regulating the biosynthesis of proresolving mediators, the latter of which may contribute to survival in murine polymicrobial sepsis.

The inflammasome is a molecular platform to promote maturation and secretion of proinflammatory cytokines, such as IL-1 β and IL-18, in immune cells (1, 2). Cytoplasmic receptors of the NLR family are critical components of the inflammasome and interact with the apoptosis-associated speck-like protein containing CARD (ASC), which recruits the precursor form of caspase-1 (1, 2). The nucleotide-binding domain, leucine-rich repeat-containing receptor, pyrin domain-containing-3 (NLRP3) inflammasome is typically activated through a two-component pathway, involving toll-like receptor 4 ligand interaction (priming) followed by a second signal, such as ATP-dependent purinergic receptor (P2rx7) activation, which regulates K⁺ efflux (1, 2).

Unlike other inflammasomes, the NLRP3 inflammasome senses pathogen-associated molecular patterns and damage-associated molecular patterns (DAMPs), such as ATP and mitochondrial DNA (mtDNA) (1, 2). On sensing pathogen-associated molecular patterns and/or DAMPs, NLRP3 recruits ASC and caspase-1, leading to caspase-1 activation,

maturation and secretion of proinflammatory cytokines, such as IL-1 β and IL-18, and initiating a lytic host cell death called pyroptosis (1–4).

Sepsis remains a major public health concern, and the number of sepsis cases per year is increasing because of the aging population, the increased longevity of people with chronic diseases, the spread of antibiotic-resistant organisms, and the broader use of immunosuppressive and chemotherapeutic agents (5, 6). Although inflammatory responses observed in sepsis are important for host defense against invading microbes, the excessive inflammation often causes severe cell and tissue damage and organ dysfunction, leading to death (5–7). Therefore it is critical to identify the cellular and immune pathways needed to mitigate the proinflammatory responses during sepsis.

Lipid-derived mediators (LMs) play key roles as signaling molecules to regulate acute inflammation (8). LMs are enzymatically produced via specific cyclooxygenase (COX) and lipoxygenase (LOX) pathways (9–11). During the onset of the resolution phase, eicosanoid class switching occurs through changes in production within the arachidonate-derived family, for example, in lipoxins (LXs) (12). LXs generated by 15-lipoxygenase type 1, specifically LXA₄ and LXB₄, promote resolution of inflammation by reducing the entry of polymorphonuclear neutrophils (PMNs) to sites of inflammation and stimulating the clearance of apoptotic neutrophils by macrophages (13, 14). This class of antiinflammatory and proresolving LMs is coined as specialized proresolving mediators (SPMs) (8), and is essential for maintaining tissue homeostasis. Although the protective roles of SPMs are observed in several inflammatory disease models, the precise intracellular molecular mechanisms by which biosynthesis of SPMs are regulated in hosts or cells remains to be established.

In this study, we describe functional roles of the NLRP3 inflammasome in the regulation of LMs and proinflammatory cytokine production in a murine model of sepsis. We also demonstrate that caspase-7 is essential for NLRP3-dependent repression of LXB₄ synthesis, and that LXB₄ increased via genetic deletion of *Nlrp3* or *Casp7* may contribute to the amelioration of inflammation and mortality of sepsis. Some of the results of these studies have been previously reported in the form of abstracts (15, 16).

Methods

Animals

Nlrp3^{−/−} mice were obtained from Dr. Richard A. Flavell (Yale University, New Haven, CT) and were backcrossed 10 times against C57BL/6 (17). *Asc*^{−/−} mice were obtained from Dr. Kate Fitzgerald (University of Massachusetts Medical School, Worcester, MA) (18). *P2rx7*^{−/−}, *Casp7*^{−/−}, and *Il18*^{−/−} mice were purchased from Jackson Laboratory (Bar Harbor, ME) (19–21).

Cecal Ligation and Puncture Model of Polymicrobial Sepsis

The cecal ligation and puncture (CLP) model of polymicrobial sepsis was performed with 10- to 12-week-old C57BL/6J wild-type male mice and *Nlrp3*^{−/−}, *Asc*^{−/−}, *P2rx7*^{−/−}, *Casp7*^{−/−}, and *Il18*^{−/−} mice (17–21). The mouse cecum was ligated below the ileocecal valve. After ligation, the cecum was punctured once with a 23-gauge needle. Sham-operated mice underwent the same procedure, including opening of the peritoneum and exposing the bowel. No antibiotics were administered to the mice after surgery to assess the systemic inflammation based on polyintestinal bacteria (22, 23). Preoperatively and postoperatively, all mice had unlimited access to food and water.

Therapeutic Administration of LXB₄

Some wild-type mice were injected with LXB₄ intravenously 10 minutes before CLP surgery. The dose was chosen based on previous studies showing the protective effects of other SPMs, such as RvD2 (24) and LXA₄ (25) (1 μ g/mouse).

Sample Extraction and Lipid Mediator Metabololipidomics

All samples for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis were extracted using solid-phase extraction columns as described previously (26). Identification was conducted using published criteria with at least six diagnostic ions (23). Additional detail on the methods for metabololipidomics is provided in the online supplement.

Statistics

Data are expressed as mean $\pm$ SEM. For comparisons between two groups,

we used two-tailed unpaired Student's *t* test or Mann-Whitney test. In experiments with more than two groups statistical differences were tested using a one-way analysis of variance followed by a Tukey test. The log-rank test was used to assess for differences in survival. *P* values of less than 0.05 were considered statistically significant.

Study Approval

All animals were housed in accordance with the guidelines from the American Association for Laboratory Animal Care. All animal experimental protocols were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee and the Animal Research Committee of Brigham and Women's Hospital.

Results

NLRP3 Deficiency Promotes Resolution of Inflammation and Enhances Survival in an Experimental Sepsis Model

To investigate the role of the NLRP3 inflammasome in systemic acute inflammation *in vivo*, we first determined the effects of NLRP3 inflammasome-associated molecules on the survival rate of mice subjected to CLP, a murine polymicrobial sepsis model. The NLRP3 deficient mice (*Nlrp3*^{-/-}) were more resistant to CLP-induced lethality than C57BL/6J wild-type mice (Figure 1A). Similarly, ASC deficient mice (*Asc*^{-/-}) and P2rx7-deficient mice (*P2rx7*^{-/-}) were more resistant to CLP-induced lethality than wild-type mice (see Figures E1A and E1B in the online supplement). We also examined the role of IL-18, a representative inflammasome-mediated cytokine, on CLP-induced mortality. Interestingly, deficiency of IL-18 did not affect the mortality of mice after CLP (see Figure E2).

Next, we evaluated the effect of NLRP3 inflammasome on tissue injury and severity of animals with sepsis. Levels of circulating cell-free mtDNA, a mitochondrial DAMP associated with mortality and severity in patients with sepsis (27–29), were increased 24 hours after CLP in wild-type mice (Figure 1B). The protein concentration of bronchoalveolar lavage fluid (BALF) and peritoneal lavage fluid, reflecting vascular

permeability, were increased in wild-type mice after CLP (Figure 1C). In contrast, *Nlrp3*^{-/-} mice displayed reduced levels of circulating mtDNA and protein concentration in both fluids, relative to wild-type mice (Figures 1B and 1C). We also observed that *Nlrp3* deficiency significantly reduced the amount of live aerobic bacteria in blood at 24 hours after CLP, compared with wild-type mice subjected to CLP (Figure 1D). To examine the functional roles of NLRP3 on inflammatory responses in CLP-induced sepsis, we measured proinflammatory cytokines in plasma from *Nlrp3*^{-/-} mice after CLP. We observed that the plasma levels of cytokines, such as IL-1 β , tumor necrosis factor- α , and IL-6, were decreased in *Nlrp3*^{-/-} mice, relative to wild-type mice (Figure 1E). Similarly the plasma levels of the antiinflammatory cytokine IL-10 were also decreased in *Nlrp3*^{-/-} mice after CLP, relative to wild-type mice (see Figure E3).

Infiltration of leukocytes into the inflammatory site is one of the important inflammatory events in the pathogenesis of sepsis. Macrophage inflammatory protein (MIP)-2, a potent chemoattractant, was increased in plasma (Figure 1F) and BALF (see Figure E4), and the number of total leukocytes and PMNs was increased in the peritoneum of wild-type mice after CLP (Figures 1G and 1H). In contrast, *Nlrp3* deficiency decreased the levels of MIP-2 in plasma and BALF of mice subjected to CLP, relative to wild-type mice (Figures 1F; see Figure E4). Consistent with the suppression of MIP-2 production, the recruitment of leukocytes including PMNs was also suppressed in *Nlrp3*^{-/-} mice after CLP (Figures 1G and 1H). These results indicate that deficiency of *Nlrp3* reduced inflammatory responses and mortality in CLP-induced sepsis.

To examine the possibility that the protective phenotype observed in *Nlrp3*^{-/-} mice after CLP may be related to changes in the virulence of colonic bacteria, we performed cecal slurry (CS) experiments. Similar to the CLP model, CS caused dose-dependent sepsis mortality when administered by intraperitoneal injection to wild-type mice (see Figure E5A) (30). CS derived from *Nlrp3*^{-/-} mice caused comparable but significantly increased sepsis mortality relative to CS derived from wild-type mice when administered at a

lethal dose (see Figure E5B), suggesting that alterations in colonic flora do not directly contribute to the sepsis protection observed in *Nlrp3*^{-/-} mice.

NLRP3 Deficiency Alters the Biosynthesis of Lipid Mediators *In Vivo* and *In Vitro*

LMs play important roles as signaling molecules to regulate immune responses during early phase of acute inflammation (8). A recent study showed the involvement of NAIP5 (NLR family, apoptosis inhibitory protein 5)/NLRC4 inflammasome in LM biosynthesis (31). To address the mechanisms by which *Nlrp3* deficiency promoted resolution of inflammation in mice during CLP-induced sepsis, we performed mass spectrometry-based metabololipidomics focusing on local acting LM. We measured endogenous LMs in peritoneal lavage fluid from wild-type and *Nlrp3*^{-/-} mice subjected to CLP. LMs were profiled using LC-MS-MS-based direct matching of their physical properties in LC-MS-MS with synthetic and authentic standards (Figures 2A and 2B; see Table E1). We found that both COX and LOX pathways were regulated after CLP, with a time-dependent increase in prostaglandins and proresolving mediators (Figure 2C) including prostaglandins E₂, LTB₄, RvD1, RvD2, RvD5, and LXB₄ (Figure 2D) in wild-type mice during the early inflammatory phase (6–12 h after CLP). In *Nlrp3*^{-/-} mice, proinflammatory LMs levels, such as LTB₄, were lower than those in wild-type mice 12 hours after CLP (Figures 2C and 2D). In contrast, SPMs including LXA₄, LXB₄, and RvD5 were increased in peritoneal lavages from *Nlrp3*^{-/-} mice at 6 hours after CLP, relative to wild-type mice (Figure 2D). Taken together, these results suggest that *Nlrp3* deficiency enhanced survival from CLP-induced sepsis by up-regulating SPMs and down-regulating proinflammatory LMs. Of note, *Nlrp3* deficiency up-regulated SPMs, such as LXB₄, in mice subjected to CLP to levels that reached statistical significance, before proinflammatory LMs were down-regulated (Figure 2D; see Table E1).

To assess the contribution of macrophages, critical cells in acute inflammation and inflammasome activation, to the biosynthesis of LMs, we investigated the LM profiles of bone marrow-derived macrophages (BMDMs)

Figure 1. NLRP3 deficiency promotes resolution of inflammation and enhances survival in cecal ligation and puncture (CLP)-induced sepsis. (A) *Nlrp3*^{-/-} or wild-type control (C57BL/6J) mice were subjected to CLP surgery. Rates of survival were determined for *Nlrp3*^{-/-} mice after CLP (n = 10 per condition). Two independent experiments were performed (total n = 20 for each strain), and the representative survival curve is shown. **P* < 0.0001 by log-rank test versus CLP wild-type mice. (B) Cell-free mitochondrial DNA in plasma from mice 24 hours after CLP and sham surgery was detected with cyclooxygenase I (COXI) primer via SYBR Green–based quantitative polymerase chain reaction. (C) Vascular permeability was validated by both bronchoalveolar lavage fluid and peritoneal lavage fluid protein concentration. (D) For determination of CFU, blood was serially diluted and then plated on LB plates. The LB plates were incubated for 24 hours at 37°C. The data represent the mean ± SEM. **P* < 0.01 by two-tailed Mann-Whitney *U* test. n = 15 for wild-type mice and n = 10 for *Nlrp3*^{-/-} mice. (E) Plasma cytokine levels. IL-1β, tumor necrosis factor-α, and IL-6 were measured by ELISA in plasma from wild-type and *Nlrp3*^{-/-} mice 24 hours after CLP, respectively. (F) Macrophage inflammatory protein-2 was measured by ELISA in plasma from wild-type and *Nlrp3*^{-/-} mice after CLP. (G and H) Total peritoneal leukocyte number and the percentage of polymorphonuclear cells in peritoneal lavage fluid (B, C, and E–H). All data represent the mean ± SEM (n = 3–8). **P* < 0.05 by unpaired, two-tailed Student's *t* test (B, C, and E–H) versus sham wild-type mice; ***P* < 0.05 by unpaired, two-tailed Student's *t* test (B, C, and E–H) versus CLP wild-type mice. BALF = bronchoalveolar lavage fluid; MIP = macrophage inflammatory protein; NLRP3 = nucleotide-binding domain, leucine-rich repeat-containing receptor, pyrin domain-containing-3; PMN = polymorphonuclear neutrophil; TNF = tumor necrosis factor.

during NLRP3 inflammasome activation. We preincubated BMDMs with LPS and then stimulated with ATP. ATP-driven activation of caspase-1 in LPS-primed

macrophages is an established model for NLRP3 inflammasome activation *in vitro*, which is mediated by P2rx7 and toll-like receptor 4 (32, 33). We compared LM

profiles between BMDMs from wild-type and *Nlrp3*^{-/-} mice during NLRP3 inflammasome activation. LMs were profiled using LC-MS-MS–based direct

Figure 2. NLRP3 deficiency alters lipid mediators' profiles during cecal ligation and puncture (CLP)-induced sepsis. Peritoneal lavages were collected from wild-type and *Nlrp3*^{-/-} mice 6 and 12 hours after CLP. Methanol was added, and lipid mediators (LM) were investigated by LM-metabolipidomics. (A) Representative multiple reaction monitoring traces obtained for the lipoxins, prostanoids, leukotrienes, D-series resolvins, protectins maresins, and E-series resolvins. (B) Representative tandem mass spectrometry spectra used for the identification of RvD1 and lipoxin B₄ (LXB₄). (C) LM time course for lipoxins (LXA₄ and LXB₄), prostaglandins (PGD₂, PGE₂, and PGF_{2α}), leukotrienes (LTB₄, Δ6-trans-LTB₄, 12epi, Δ6-trans-LTB₄, and 5S,12S-diHETE), resolvins (RvD1–6), maresins (MaR1 and 7S,14S-diHdHA), protectins (PD1 and 10S,17S-diHdHA), and E-series resolvins (RvE1–3). (D) LM time course of PGD₂, PGE₂, TXB₂, LTB₄, LXA₄, LXB₄, RvD1, RvD2, and RvD5. Bioactive LM were quantified using multiple reaction monitoring (see METHODS for details). Result for A and B are representative of n = 15 mice. Results for C and D are expressed as pg/lavage; mean ± SEM; n = 3–5 mice per group. *P ≤ 0.05, **P < 0.01, ***P < 0.001 versus respective sham mice; #P ≤ 0.05, ##P ≤ 0.01 versus respective 6-hour CLP mice; †P ≤ 0.05, ††P ≤ 0.001 versus CLP wild-type mice. LX = lipoxin.

matching of their physical properties in LC-MS-MS with synthetic and authentic standards (see Figure E6). We found that inflammasome activation by LPS and ATP up-regulated the COX and LOX biosynthetic pathways in wild-type and *Nlrp3*^{-/-} macrophages (Figure 3A; see Table E2). Of note in these incubations we found marked and statistically significant increases in the levels of the proresolving mediator LXB₄ within 30 minutes of ATP treatment in LPS-primed *Nlrp3*^{-/-} BMDMs when compared with wild-type BMDMs (Figure 3B). These findings are similar with those observed in peritoneal lavages from mice subjected to CLP (Figure 2). ATP had no effect on LXB₄ biosynthesis in both wild-type and *Nlrp3*^{-/-} cells by itself (Figure 3B). These results suggest that the NLRP3 inflammasome uniquely regulates the biosynthesis of the proresolving mediator LXB₄ in macrophages.

LXB₄ Enhances Survival in CLP-induced Sepsis

LXB₄ is biosynthesized in leukocytes from arachidonic acid generated through activation of cytosolic phospholipase A2 (Figure 4A). LXB₄ is particularly synthesized via two distinct lipoxygenation steps: first by lipoxygenation at the 15 position producing 15-hydroperoxyeicosatetraenoic acid that is then converted to LXB₄ by subsequent lipoxygenation and epoxidation steps (Figure 4A; see Figure E7) (34). Although LXB₄ displays potent antiinflammatory properties as a SPM (35), its precise roles in the pathogenesis of sepsis remain unclear. To examine the roles of LXB₄ in polymicrobial sepsis, mice were injected with LXB₄ intravenously and subjected to CLP. We observed that the number of total infiltrated leukocytes (Figure 4B) and PMNs that account for approximately 80% of the infiltrated leukocytes in peritoneum (Figure 4C) was decreased in LXB₄-treated mice subjected to CLP, relative to vehicle-treated mice. Consequently, LXB₄ treatment enhanced the survival rate of the mice subjected to CLP (Figure 4D). These data suggest that LXB₄ may be a critical molecule to mediate the protective effect of *Nlrp3* deficiency in CLP-induced sepsis.

NLRP3 Inflammasome-mediated Caspase-7 Activation Regulates LXB₄ Production in Macrophages

We next assessed the mechanism by which NLRP3 regulated LXB₄ synthesis *in vivo* and *in vitro*. Recent studies suggest that caspase-7 is activated by the inflammasome (36), and is involved in the susceptibility of mice to LPS-induced septic shock (37). We first tested whether caspase-7 is regulated by NLRP3 inflammasome activation in macrophages. The cleaved form of caspase-7 was increased by LPS and ATP in wild-type macrophages (Figure 5A) (36). However, the expression of cleaved caspase-7 did not increase in response to LPS and ATP in *Nlrp3*^{-/-} BMDMs (Figure 5A), suggesting that the NLRP3 inflammasome regulates caspase-7 activation.

We next investigated the correlation of caspase-7 with caspase-1 activation using *Casp7* deficient BMDMs. Deficiency of *Casp7* did not affect basal levels of ASC and P2rx7 and LPS-induced NLRP3 expression. In addition, caspase-1 activation was comparable between *Casp7* deficient cells and the wild-type cells in response to LPS and ATP (Figure 5B) (36). Because the roles of caspase-7 on cell death remains unclear, we also examined the effect of caspase-7 on pyroptosis, a programmed cell death in immune cells during inflammation, by assessing lactate dehydrogenase (LDH) release. The secretion of LDH was increased in BMDMs during NLRP3 inflammasome activation (Figure 5C). Increased LDH secretion was suppressed in *Nlrp3*^{-/-}, *Asc*^{-/-}, and *P2rx7*^{-/-} BMDMs, compared with wild-type cells in response to LPS and ATP (Figure 5C; see Figure E8). *Casp7* deficiency also suppressed LDH secretion in macrophages during NLRP3 inflammasome activation (Figure 5D).

Because the LXB₄ biosynthesis and activation of caspase-7 were regulated by NLRP3 inflammasome activation (Figures 3B and 5A), we next assessed the role of caspase-7 on LXB₄ production during NLRP3 inflammasome activation. Importantly, we found an increase in the levels of the proresolving mediator LXB₄ in *Casp7*^{-/-} BMDMs during NLRP3 inflammasome activation compared with wild-type BMDMs (Figure 6A). Furthermore, exogenous treatment of LXB₄ suppressed LDH secretion in

BMDMs in response to LPS and ATP (Figure 6B), which may explain the antipyroptotic effect of caspase-7 deficiency. These findings are in accord with those observed in *Nlrp3*-deficient macrophages treated with LPS and ATP (Figure 3). Thus, our results suggest that the increased generation of LXB₄ in *Nlrp3*^{-/-} cells is mediated by suppression of caspase-7 activation. In addition, these data suggest that the NLRP3 inflammasome promotes pyroptosis by caspase-7-mediated inhibition of LXB₄ synthesis in macrophages.

Caspase-7 Deficiency Promotes Resolution of Inflammation and Enhances Survival in Sepsis

We also tested the *in vivo* relevance of caspase-7 in polymicrobial sepsis. *Casp7*^{-/-} mice were more resistant to CLP-induced lethality than wild-type mice (Figure 7A). Furthermore, the protein concentrations of both BALF and peritoneal lavage fluid were decreased in *Casp7*^{-/-} mice relative to wild-type mice after CLP (Figures 7B and 7C). Plasma levels of proinflammatory cytokines, such as tumor necrosis factor- α and IL-6, were also decreased in *Casp7*^{-/-} mice subjected to CLP, relative to wild-type mice (Figure 7D). We also observed that the levels of MIP-2 in BALF were also decreased in *Casp7*^{-/-} mice after CLP (see Figure E9).

Because caspase-7 is involved in pyroptosis during NLRP3 inflammasome activation (Figure 5D) (36), we also assessed whether caspase-7 regulates macrophage cell death during CLP-induced sepsis. Cells in peritoneal fluid from *Casp7*^{-/-} mice were stained with CD11b, a marker of leukocytes, and propidium iodide, a fluorescent membrane-impermeant molecule used for identifying dead cells. The number of CD11b- and propidium iodide-positive cells was increased in wild-type mice after CLP (Figure 7E), whereas there was no difference in the number of CD11b- and propidium iodide-positive cells between *Casp7*^{-/-} mice subjected to either sham operation or CLP (Figure 7E). The number of CD11b- and propidium iodide-positive cells was also lower in *Casp7*^{-/-} mice than wild-type mice after CLP (Figure 7E).

To address the mechanisms by which *Casp7* deficiency rescued mice from CLP-induced sepsis, we analyzed the levels of

Figure 3. NLRP3 deficiency alters biosynthesis of lipid mediators *in vitro*. The bone marrow-derived macrophages (BMDMs) isolated from *Nlrp3*^{-/-} mice and wild-type mice were incubated with vehicle, LPS (6.5 h), ATP (0.5 h), or with LPS (6 h) followed by ATP (30 min) for NLRP3 inflammasome activation. After stimulation by LPS and ATP, lipid mediators (LM) were investigated by LM-metabololipidomics. (A) LM time course of prostaglandins (PG), LTB₄ metabolome, lipoxins, E- and D-series resolvins, maresins, and protectins families. (B) LM time course of PGD₂, PGE₂, TxB₂, LTB₄, LXA₄, LXB₄, RvD1, RvD2, and RvD5. Bioactive LM were quantified using multiple reaction monitoring (see METHODS for details). Results for A and B are expressed as mean ± SEM. Results are representative of five experiments. **P* ≤ 0.05, ***P* < 0.01, ****P* < 0.001 versus respective vehicle incubations; #*P* ≤ 0.05, ##*P* < 0.01, versus wild-type BMDM + LPS + ATP. LX = lipoxin; NLRP3 = nucleotide-binding domain, leucine-rich repeat-containing receptor, pyrin domain-containing-3.

Figure 4. Lipoxin B₄ (LXB₄) enhances survival in cecal ligation and puncture (CLP)-induced sepsis. (A) LXB₄ biosynthetic pathway in leukocytes. (B and C) Total peritoneal leukocytes number and the percentage of polymorphonuclear cells in peritoneal lavage fluid. All data represent mean $\pm$ SEM; n = 3 mice in sham groups and n = 6 mice in CLP groups. [‡]P < 0.05 by unpaired, two-tailed Student's *t* test (B and C) versus vehicle-treated sham mice; [§]P < 0.05 by unpaired, two-tailed Student's *t* test (B and C) versus vehicle-treated CLP mice. (D) Wild-type mice were intravenously administered with LXB₄ (1 μ g/mouse) or vehicle 10 minutes before CLP surgery (n = 10 per condition). Rate of survival was determined for mice treated with LXB₄ after CLP. *P < 0.05 by log-rank test versus vehicle-treated CLP mice. PMN = polymorphonuclear neutrophil.

local acting LM by performing mass spectrometry-based metabololipidomics. We measured endogenous LMs in peritoneal lavage fluid from wild-type and *Casp7*^{-/-} mice subjected to CLP (Figures 8A and 8B; see Table E3). Similar with those observed in *Nlrp3*^{-/-} mice, proresolving mediator levels including LXB₄ were found to be significantly elevated in peritoneal lavages from *Casp7*^{-/-} mice when compared with wild-type mice (Figure 8B). In addition, the levels of proinflammatory LMs including LTB₄ and TXB₂ were significantly reduced in these mice

(Figure 8B). Taken together, similar to NLRP3, caspase-7 is also involved in inflammatory responses and the susceptibility of CLP-induced sepsis by regulating LM biosynthesis.

Critical Roles of LOX Activity in NLRP3 Deficiency during Sepsis

Finally, we examined whether increased production of endogenous LXB₄ in *Nlrp3*^{-/-} mice contributed to the survival of sepsis by administering baicalein, a potent LOX inhibitor (38). We observed that baicalein treatment significantly increased the mortality of *Nlrp3*^{-/-}

mice after CLP, compared with *Nlrp3*^{-/-} mice treated with vehicle (Figure 9). This result suggests that the mechanism by which *Nlrp3* deficiency rescued the mice from sepsis is mediated by increased synthesis of lipoxins.

Discussion

Physiologic resolution, namely the termination of acute inflammatory responses, is critical to maintain host homeostasis in response to infection (38). This acute self-limiting response is characterized by active signaling molecules called SPMs that limit further inflammatory cell recruitment and enhance the phagocytosis of macrophages (39). In this report, we demonstrate that NLRP3 inflammasome-related molecules (e.g., NLRP3 and ASC) are critically involved in the pathogenesis of CLP-induced polymicrobial sepsis by augmenting acute inflammation and tissue injury. The mechanism by which *Nlrp3* deficiency rescued septic mice was mediated by regulating the biosynthesis of LMs. We suggest that endogenous SPM (i.e., LXB₄) synthesis is regulated by NLRP3 inflammasome-mediated caspase-7 activation and is a critical cytoprotective mechanism during CLP-induced sepsis, based on the following observations: LXB₄ synthesis is increased in leukocytes of *Nlrp3*- or *Casp7*-deficient mice at the earlier phase during sepsis. Second, deficiency of *Nlrp3* or *Casp7* reduced the susceptibility of mice to CLP-induced sepsis, accompanied by enhanced SPMs synthesis including LXB₄, but reduced proinflammatory mediators. LXB₄ treatment improved the survival of CLP-induced sepsis. Finally, pharmacologic inhibition of LOX, a critical enzyme for LXB₄ synthesis, increased susceptibility to CLP-induced sepsis in *Nlrp3*^{-/-} mice.

Although several studies show that exogenous administration of SPMs, such as RvD2 and LXA₄, or an antagonist for proinflammatory LMs rescues CLP-induced septic mice (24, 25), it remains unclear how LMs are endogenously synthesized or regulated during CLP-induced sepsis. A previous study provides a link between the NAIP5/NLRC4 inflammasome and biosynthesis of

Figure 5. Activation of caspase-7 during NLRP3 inflammasome activation contributes to pyroptosis. (A) Immunoblot analysis for caspase-1, NLRP3, and caspase-7 of cell lysates from wild-type and *Nlrp3*^{-/-} bone marrow-derived macrophages (BMDMs) stimulated with LPS and ATP. β-Actin served as the standard. Data are representative of three experiments. (B) Immunoblot analysis for caspase-1 in cell lysates from wild-type and *Casp7*^{-/-} BMDMs stimulated with LPS and ATP. Data are representative of three experiments. (C) Measurement of cell-free lactate dehydrogenase (LDH) in cell culture media from wild-type and *Nlrp3*^{-/-} BMDMs stimulated with LPS and ATP. (D) Measurement of cell-free LDH in cell culture media from wild-type and *Casp7*^{-/-} BMDMs stimulated with LPS and ATP. (B and D) Data are representative of two to three experiments. All data represent the mean ± SEM. **P* < 0.05 by unpaired, two-tailed Student's *t* test versus wild-type control group; #*P* < 0.05 by unpaired, two-tailed Student's *t* test versus wild-type LPS and ATP treatment group. ASC = apoptosis-associated speck-like protein containing CARD; NLRP3 = nucleotide-binding domain, leucine-rich repeat-containing receptor, pyrin domain-containing-3; P2x7R = P2X7 receptor.

proinflammatory mediators, such as prostaglandin E₂ (31); however, the regulatory role of NLRP3 inflammasome in resolution of inflammation remains unclear. Our study demonstrates not only NLRP3 inflammasome-mediated LMs regulation, but also a unique role of NLRP3 in SPMs regulation and the resolution of inflammation through caspase-7 activation.

Increase of SPMs (e.g., LXB₄) observed at 6 hours after CLP was likely to be an

early event to initiate the resolution of CLP-induced inflammation in *Nlrp3*^{-/-} and *Casp7*^{-/-} mice, because the levels of proinflammatory LMs, such as LTB₄ or TXB₂, were significantly reduced at 12 hours after CLP in those mice. In addition, subsequent to the reduction of proinflammatory LMs, *Nlrp3*^{-/-} and *Casp7*^{-/-} mice displayed less vascular endothelial permeability, recruitment of proinflammatory cells and proinflammatory cytokine production at

24 hours after CLP. Furthermore, *Nlrp3*^{-/-} mice also produced less antiinflammatory cytokine IL-10, suggesting the resolution of inflammation by NLRP3 deficiency was unlikely to be mediated by IL-10. The phenotype of *Nlrp3*^{-/-} mice was not likely related to changes in the virulence of the colonic flora because CS from these mice caused slight increase in sepsis mortality relative to CS derived from control wild-type mice when administered to wild-type mice.

Figure 6. Caspase-7 deficiency promotes lipoxin B₄ (LXB₄) synthesis, which protects cells from pyroptosis. (A) LXB₄ levels in wild-type and Casp7^{-/-} bone marrow-derived macrophages stimulated with LPS and ATP was quantified by lipid mediator metabololipidomics. (B) Measurement of cell-free LDH in cell culture media from wild-type bone marrow-derived macrophages stimulated with LPS and ATP in the presence of LXB₄. (A and B) Data are representative of two to three experiments. All data represent the mean $\pm$ SEM. * $P < 0.05$ by unpaired, two-tailed Student's t test versus wild-type control group; # $P < 0.05$ by unpaired, two-tailed Student's t test versus wild-type LPS and ATP treatment group. LDH = lactate dehydrogenase.

We also observed that SPM-mediated resolution of inflammation dampened the release of mitochondrial DAMPs (i.e., cell-free circulating mtDNA). Thus, early enhancement of SPMs before suppression of proinflammatory LMs in *Nlrp3*^{-/-} mice suggests that *Nlrp3* deficiency promotes the timely resolution of the inflammatory response by increased biosynthesis of SPMs during CLP-induced immune responses. Our study also describes a novel function of LXB₄ in inhibiting NLRP3 inflammasome-mediated pyroptosis. Although pyroptosis is often recognized as a beneficial host-defense response to prevent growth of intracellular microbes (3), excess macrophage cell death may contribute to the release of DAMPs, which further promote inflammatory responses and tissue (organ) injuries (40, 41). We show that deficiency of *Asc*, *Nlrp3*, or *P2rx7* improved the survival of mice subjected to CLP-induced sepsis and also inhibited pyroptosis in response to LPS and ATP, suggesting that inhibition of pyroptosis may be associated with improved survival from sepsis. Further studies are needed to identify the precise roles of pyroptosis in sepsis.

We found that several SPMs including LXA₄ and RvD5 were elevated in wild-type mice after CLP, whereas the levels of those SPMs were unchanged in

wild-type BMDM-stimulated LPS and ATP. These differences observed between *in vitro* and *in vivo* experiments may reflect either differential biosynthetic origin for the mediators including different cell types or distinct routes of further metabolism. In addition, our data suggest that each SPM can be regulated by its distinctive signaling pathway. It is possible that *Nlrp3* deficiency specifically activates 15-lipoxygenase, a critical enzyme for the biosynthesis of lipoxins (Figure 4A). Other possible mechanisms may include differential metabolic rates of LXB₄ between wild-type and *Nlrp3*-deficient macrophages. For example, it is possible that in *Nlrp3*-deficient cells, LXB₄ is less susceptible to further conversion by macrophage metabolic enzymes including 15-prostaglandin dehydrogenase (42), which leads to the accumulation of LXB₄ *in vitro* and *in vivo*. The detailed mechanism by which NLRP3 regulates LXB₄ production remains to be established. Nonetheless, the present findings identify caspase-7, a downstream molecule of the NLRP3 inflammasome, as a key molecule for SPMs synthesis.

In conclusion, our results demonstrate that NLRP3-mediated LM regulation potentially contributed to inflammation and susceptibility of CLP-induced sepsis. Moreover, the NLRP3 inflammasome regulates LM synthesis by

activating caspase-7. Although it remains unclear how LXB₄-mediated antipyroptosis regulates inflammation and susceptibility to sepsis, our present results provide a novel molecular link between NLRP3, caspase-7, and endogenous LM biosynthesis. The NLRP3 inflammasome has well-known pleiotropic and potentially “double-edged sword” effects in infection as a regulator of inflammation and an integral part of the immune response. Excessive inflammasome-mediated immune responses can also potentially exacerbate tissue injury (1, 2). In this regard, our study demonstrates the beneficial roles of *Nlrp3* deficiency (i.e., inhibiting inflammation, tissue injuries, and death) in polymicrobial septic mice.

Our study has several important limitations. First, the injection of LXB₄ at a pharmacologic dose, which provided significant protection in sepsis, likely exceeded that of the measured endogenous production of this mediator, and thus does not necessarily establish the efficacy, nor recapitulate the physiologic context of naturally occurring LXB₄. Second, the pan-LOX inhibitor baicalein, which protected *Nlrp3*^{-/-} mice against CLP, is not entirely specific for 12/15 LOX, and may also affect Ca²⁺ flux. The application of baicalein in the wild-type mice displayed a trend toward sepsis

Figure 7. Caspase-7 deficiency promotes specialized proresolving mediator synthesis and protects mice from cecal ligation and puncture (CLP)-induced sepsis. (A) *Caspase-7*^{-/-} or wild-type control mice were subjected to CLP surgery. Rate of survival was determined for *Casp7*^{-/-} mice after CLP (n = 10 per condition). Two independent experiments were performed (total n = 20 for each strain) and the representative survival curve is shown. **P* < 0.05 by log-rank test versus CLP wild-type mice. (B and C) Protein concentration in bronchoalveolar lavage fluid (BALF) (B) and peritoneal lavage fluid (C) harvested from wild-type and *Casp7*^{-/-} mice 24 hours after CLP. (D) The level of plasma tumor necrosis factor-α and IL-6 were measured by ELISA. Plasma was prepared from wild-type and *Casp7*^{-/-} mice 24 hours after CLP, respectively. (B–D) All data represent the mean ± SEM; n = 3–5 mice per group. ‡*P* < 0.05 by unpaired, two-tailed Student's *t* test (B–D) versus sham wild-type mice; §*P* < 0.05 by unpaired, two-tailed Student's *t* test (B–D) versus CLP wild-type mice. (E) Cells collected by peritoneal lavage from wild-type or *Casp7*^{-/-} 24 hours after CLP were stained with propidium iodide and leukocyte marker CD11b. The x-axis shows the fluorescent signal intensity, and the y-axis represents the CD11b-positive cell number normalized as a percentage of the maximum (% of max). Data are representative of two experiments. The data represent the mean ± SEM. ****P* < 0.05 by unpaired, two-tailed Student's *t* test versus CLP wild-type mice. (n = 3–5 for wild-type mice and n = 3–5 for *Casp7*^{-/-} mice). PI = propidium iodide; TNF = tumor necrosis factor.

protection that did not reach statistical significance. Finally, the *Nlrp3*^{-/-} mice displayed partial reduction of IL-1β in the plasma in response to CLP. Previous

studies have described resistance phenotypes in IL-1β/IL-18 double knockout mice subjected to CLP (43). We have also observed in our studies a

resistance phenotype of *Il1r*^{-/-} mice (data not shown). However, *Il18*^{-/-} mice had no phenotype as shown in Figure E2. Therefore, although our data support a

A **B**

Figure 8. Caspase-7 deficiency promotes lipoxin B₄ synthesis *in vivo*. (A and B) Specific bioactive lipid mediator and precursor/pathway markers measured by multiple reaction monitoring in lavages from mice 6 and 12 hours after cecal ligation and puncture (CLP) and sham mice. (B) Time course for identified lipid mediators. Selected lipid mediator levels identified in peritoneal lavages. Results are expressed as pg/lavage; mean $\pm$ SEM; $n = 3-5$ mice per group. * $P \leq 0.05$ versus sham wild-type mice; # $P \leq 0.05$ versus *Casp7*^{-/-} sham mice; † $P \leq 0.05$ versus CLP wild-type mice. PG = prostaglandin; LX = lipoxin.

Figure 9. Inhibition of lipoxygenase activity increases mortality of *Nlrp3*^{-/-} mice in sepsis. *Nlrp3*^{-/-} mice or wild-type mice were intravenously administered with baicalein (100 µg/mouse) or vehicle 1 hour before cecal ligation and puncture (CLP) surgery. Survival curve of CLP was determined in wild-type mice with sham + vehicle (n = 3), wild-type mice with sham + baicalein (n = 3), wild-type mice with CLP + vehicle (n = 8), wild-type mice with CLP + baicalein (n = 8), *Nlrp3*^{-/-} mice with sham + vehicle (n = 3), *Nlrp3*^{-/-} mice with sham + baicalein (n = 3), *Nlrp3*^{-/-} mice with CLP + vehicle (n = 29), *Nlrp3*^{-/-} mice with CLP + baicalein (n = 25). **P* = 0.027 (between *Nlrp3*^{-/-} mice with CLP + vehicle and *Nlrp3*^{-/-} mice with CLP + baicalein); #not significant, *P* > 0.05 (between wild-type mice with CLP + vehicle and wild-type mice with CLP + baicalein), by log-rank test. NLRP3 = nucleotide-binding domain, leucine-rich repeat-containing receptor, pyrin domain-containing-3.

protective role for LXB₄, it remains possible that differences in production of IL-1β and other cytokines may contribute to the prosurvival phenotype

of *Nlrp3*^{-/-} mice during sepsis development.

Although translation of the current findings to the human condition

remains speculative, individuals with genetic predisposition to enhanced NLRP3 inflammasome responses may conceivably be at increased risk for sepsis-induced mortality. Our recent studies suggest that LM profiles were altered in critically ill patients with sepsis. We found increases in proinflammatory mediators (e.g., prostaglandin F_{2α} and leukotriene B₄) and proresolving mediators (e.g., resolvin E1, resolvin D5, and 17R-protectin D1), in sepsis nonsurvivors relative to survivors, and that altered mediator profiles were predictive of respiratory failure (44). Although our current studies suggest that LXB₄ is an important proresolving mediator in responses to sepsis in the mouse, further research is needed to evaluate the potential of LXB₄ and other SPMs as useful biomarkers of the progression of human sepsis. Given several reports showing critical roles of NLRP3 in inflammation, this unique regulation of SPMs biosynthesis by NLRP3 and caspase-7 may provide new therapeutic strategies for the excess morbidity and mortality associated with this disease and related inflammatory disorders. ■

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