3-Hydroxykynurenine Regulates Lipopolysaccharide-Stimulated IL-6 Production and Protects against Endotoxic Shock in Mice

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3-Hydroxykynurenine Regulates Lipopolysaccharide-Stimulated IL-6 Production and Protects against Endotoxic Shock in Mice

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ABSTRACT

Despite advances in our understanding of endotoxic shock, novel therapeutic interventions that can reduce the burden of sepsis remain elusive. Current treatment options are limited, and it is only through refinements in the ways that we deliver supportive care that mortality has fallen over the years. In this study, the role of kynurenine 3-monooxygenase (KMO) in immune regulation was examined in LPS-induced endotoxemia using KMO−/− and KMO+/+ mice treated with the KMO inhibitor Ro61-8048. We showed that LPS-induced or cecal ligation and puncture–induced mortality and hepatic IL-6 production increased in the absence of KMO, possibly involving increased activating transcription factor 4 (ATF4) signaling in hepatic macrophages. Moreover, treatment of septic mice with 3-hydroxykynurenine reduced mortality rates and inflammatory responses regardless of the presence or absence of KMO. According to our results, the administration of 3-hydroxykynurenine as part of the treatment approach for sepsis or as an adjuvant therapy might reduce the overproduction of IL-6, which is responsible for severe endotoxemia, and ultimately improve the survival rates of patients with sepsis. ImmunoHorizons, 2021, 5: 523–534.

INTRODUCTION

In bacterial infections, appropriate immune responses are induced in the host body to eliminate the invading microbes and maintain immunological homeostasis. However, aberrant activation of immune responses against invading microbes often causes severe inflammatory disorders that may result in tissue injury. Sepsis is a systemic inflammatory disorder caused by bacterial infections and is characterized by elevated levels of proinflammatory cytokines in the serum, including IL-6, IL-1β, and TNF-α (1), that lead to multiple organ failure and mortality (2). Despite numerous studies on the pathology of sepsis, this syndrome remains the chief cause of death in intensive care units (3). In such situations, the liver might be an important target of sepsis-related injury and may lead to systemic inflammatory responses. LPS, which is a component of Gram-negative

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M.H. and K.S. planned the study. M.H., H.K., K.N., T.A., C.T., Y.Y., and H.T. performed the experiments. M.H. and H.K. were responsible for data integrity and analyses. M.H., H.K., K.N., T.A., C.T., Y.Y., H.T., and K.S. discussed and interpreted the results. M.H. and H.T. drafted the manuscript. M.H. and K.S. conducted the study. K.S. had the primary responsibility for the final content. All authors reviewed the manuscript.

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Abbreviations used in this article: AA, anthranilic acid; ALT, alanine transaminase; ATF4, activating transcription factor 4; CLP, cecal ligation and puncture; 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; KA, kynurenic acid; KMO, kynurenine 3-monooxygenase; KMO−/−, KMO gene–deficient; KP, kynurenine pathway; KYN, kynurenine; RRID, Research Resource Identifier; TRP, tryptophan.

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bacterial cell walls, has been implicated as an important factor that promotes liver injury such as sepsis and alcoholic hepatitis (4). In this context, LPS-activated hepatic macrophages play a prominent role in promoting liver injury (5). Despite its potential critical importance, the molecular mechanism by which hepatic macrophages are activated by LPS remains largely unknown.

Kynurenine 3-monooxygenase (KMO) is a pivotal enzyme in the kynurenine pathway (KP) and normally oxidizes kynurenine (KYN) to 3-hydroxykynurenine (3-HK) in the presence of reduced NADPH and molecular oxygen. KMO is predominantly localized in the outer membrane of mitochondria and is constitutively expressed in mononuclear phagocytes such as macrophages, and its expression is upregulated by IFN-γ (6, 7). KMO inhibition is thought to have beneficial effects in several instances. For example, the inhibition of KMO ameliorates neurodegenerative disorders such as Alzheimer disease and Huntington disease (8, 9). Furthermore, the absence of KMO ameliorates symptoms in acute viral myocarditis, acute pancreatitis–induced multiorgan dysfunction syndrome, and acute kidney allograft rejection (10–12). Interestingly, tryptophan (TRP) metabolites, including KYN, 3-HK, 3-hydroxanthranilic acid (3-HAA), and quinolinic acid, induce effector T cell apoptosis or regulatory T cell development in vitro, thereby regulating T cell–dependent immune responses (13, 14). KP is known to regulate inflammation and oxidative stress during sepsis (15). However, whether the induction of KMO has beneficial effects and how it regulates the immune response in the host remains unclear.

In this study, we examined the role of KMO in immune regulation in LPS-induced sepsis using KMO gene–deficient (KMO−/−) and KMO+/+ mice treated with Ro61-8048, a competitive high-affinity KMO inhibitor.

**MATERIALS AND METHODS**

**Materials**

LPS from *Salmonella enterica* (serotype abortus equi), *Escherichia coli* (serotype O55:K5), l-TRP, l-KYN, kynurenic acid (KA), ananthranilic acid (AA), 3-HAA, 3-HK, and the KMO inhibitor Ro61-8048 were purchased from Sigma-Aldrich (St. Louis, MO).

**Experimental animals**

We used 8-wk-old male mice in this study. KMO−/− mice on a C57BL/6N background were obtained from the Knockout Mouse Project repository (https://www.komp.org/redirect.html). Homozygous KMO−/− and KMO+/+ mice were generated by intercrossing heterozygous mice and genotyped using standard PCR-based genotyping of genomic DNA extracted from tail snippets. The following primer sequences were used for PCR genotyping: KMO gene sense, S′-TTCTGACCCCATCTGTGCTTCTCC-3′, and antisense, S′-ATCGAGCTCTCTAAATATGGTGGC-3′; and KMO gene deficiency sense, S′-AACCTTCACCCCTTCCAC-3′, and antisense, S′-GACCACCTCATAAGGAGC-3′. The mice were housed in a specific pathogen–free environment at our animal facility. All experiments were performed in accordance with the Guidelines for Animal Care of Fujita Health University. Mice were housed in plastic cages (five mice per cage) under controlled conditions of light (12/12-h light/dark cycle) and temperature (23 ± 2°C) and had free access to food and water. The protocols for all animal experiments were approved by the Animal Experimentation Committee of Fujita Health University (approval no. API7018). Procedures involving mice and their care conformed to international guidelines, as described in the Principles of Laboratory Animal Care (National Institutes of Health publication 85–23, revised 1985). A total of 100 KMO−/− and KMO+/+ mice were used for the in vivo experiments.

**LPS-induced endotoxemia mouse model**

To induce endotoxic shock, KMO−/− and KMO+/+ mice (n = 6) were injected i.p. with PBS or LPS (dissolved in PBS, 15 mg/kg, single injection). Cohoused mice from the same litter were randomly allocated to different treatment groups prior to the start of the experiment to avoid subjective bias in allocating mice into treatment groups after symptom onset. The investigators were blinded to the identity of the treatment groups until data analysis was performed. Mortality after LPS injection was monitored for 6 d in both KMO−/− (n = 31) and KMO+/+ (n = 30) mice. To obtain samples, the animals were anesthetized and humanely euthanized at the indicated times. The time of LPS injection was defined as 0 h in the subsequent experiments.

To block KMO activity, mice (n = 9) were injected i.p. with the KMO inhibitor Ro61-8048 (50 mg/kg) for 12 to 36 h before LPS injection. 3-HK was administered as described previously (16). To evaluate its effects, 10 mg/kg of 3-HK was administered i.p. every 12 h after LPS injection. Mortality after LPS injection was monitored for 6 d in KMO−/− (n = 30) and KMO+/+ (n = 31) mice (n = 30) and 3-HK–treated KMO−/− mice (n = 30), KMO+/+ mice (n = 30), and 3-HK–treated KMO+/+ mice (n = 30).

**Mouse sepsis model**

Sepsis was induced by cecal ligation and puncture (CLP), as previously reported (17). Briefly, mice were anesthetized, followed by midline incision (1 cm) on the anterior abdomen. The cecum was exposed and ligated below the ileocecal junction without causing bowel obstruction. A single puncture was performed using a 22-gauge needle to induce septic injury. Pressure was applied to express the cecum contents through the punctures. The cecum was placed back in the abdominal cavity, and the peritoneal wall and skin incision were closed. Sham-operated animals underwent an identical laparotomy without CLP. Mortality after CLP was monitored in KMO−/− mice (n = 30), KMO+/+ mice (n = 30), and vehicle-treated mice (n = 30). To obtain samples, the animals were anesthetized and humanely euthanized at the indicated times.

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**Measurement of serum biochemical parameters**

Blood was collected from the abdominal vena cava and centrifuged at 3000 × g for 10 min at 24°C to obtain serum. Serum alanine transaminase (ALT) levels were determined using the BioMajesty JCA-BM 2250 clinical biochemistry automatic analyzer (Japan Electron Optics Laboratory, Tokyo, Japan).

**Measurements of serum cytokine and NO levels**

IL-6 and NO in serum samples were measured using a mouse IL-6 ELISA kit (eBioscience, San Diego, CA) and an ultrasoni-cet colorimetric Nitric Oxide Synthase Assay Kit (Oxford Bio-medical Research, Rochester Hills, MI), respectively.

**Measurement of KMO activity**

KMO activity in the liver and lung tissues was determined as previously described (18). In brief, these tissues were homoge-nized in ice-cold 0.32 M sucrose. Subsequently, the homogenate was centrifuged at 7000 × g for 10 min at 4°C. The pellet was washed twice with ice-cold 0.32 M sucrose and then resuspended and sonicated in ice-cold 0.14 M potassium chloride/20 mM potassium phosphate buffer (pH 7). The reaction mixture consisted of 50 μl of enzyme preparation and 50 μl of the sub-strate solution. The reaction mixture comprised 1.1 M potassium phosphate buffer (pH 7.5), 4 mM magnesium chloride, 3 mM glucose 6-phosphate, 0.4 U of glucose-6-phosphate dehydrogenase, 0.8 mM NADP, and 2 mM L-KYN. After incubating the reaction mixture at 37°C for 60 min, the samples were acidified with 3% perchloric acid and centrifuged at 7000 × g for 10 min at 4°C. The amount of 3-HK in the reaction mixtures with and without 60-min incubation for each sample was measured using HPLC (see Measurement of KP metabolites). The amount of 3-HK formed enzymatically was calculated for each sample by subtracting the amount of 3-HK determined without incubation from that determined after 60-min incubation. This enzyme activity is expressed as the amount of 3-HK formed per hour per milligram of protein. Proteins in the liver or lung samples were assayed by the Bradford method using BSA as standard.

**Histological and immunohistochemical analyses**

Livers and lungs were fixed in 10% formalin in PBS overnight and then embedded in paraffin. Sections (4-μm thickness) were used for H&E staining and KMO and F4/80 immuno-staining, as described previously (19). The primary Abs used were rabbit polyclonal anti-KMO Ab (catalog no. 10698-1-AP, Research Resource Identifier [RRID]: AB_2296744; Proteintech Group, Rosemont, IL) and rat monoclonal anti-F4/80 Ab (catalog no. ab16911, RRID: AB_443548; Abcam) in 1% BSA in PBS overnight at 4°C. Negative controls (without primary Abs) were included for each setup. After primary Ab incubation, the sections were rinsed with PBS and incubated with secondary Abs for 30 min at room temperature. The secondary Abs used were donkey anti-rabbit IgG (H+L) Ab (catalog no. N0104, RRID: AB_623676; R&D Systems, Minneapolis, MI) and goat anti-rat IgG (H+L) Ab (catalog no. ab150157, RRID: AB_272251; Abcam), and nuclei were stained with DAPI (Dojindo Molecular Technologies, Tokyo, Japan). Immunostained slides were observed under a fluorescence microscope BX 51 equipped with a DP74 digital camera (Olympus).

**Measurement of KP metabolites**

TRP, KYN, KA, AA, and 3-HAA levels were measured as previ-ously described (10, 19). In brief, tissues (liver and lung) were homogenized (1:3, w/v) in 0.13% perchloric acid. Fifty microliters of the resulting supernatant was subjected to HPLC analysis (Shimadzu Corporation, Kyoto, Japan). Moreover, 3-HK was detected electrochemically using an ECD 300 detector (oxida-tion potential: +0.05 V; Eicom, Kyoto, Japan).

**Isolation of F4/80+ cells**

The mice were euthanized at 0 and 6 h after LPS administra-tion. Peritoneal cavity cells were harvested by injecting 10 ml ice-cold PBS into the peritoneal cavity. The collected cells were centrifuged at 800 × g for 5 min, the supernatant was discarded, and the pellet was resuspended for counting. The lungs and livers were isolated from PBS-perfused mice, chopped finely, dissociated, and incubated for 45 min with collagenase type I solution (100 U/ml in HBSS) in a shaking incubator at 37°C. After incubation, single-cell suspensions were filtered through a 70-μm filter. Lung single cells were collected and centrifuged at 800 × g for 5 min. Liver single cells were collected and centrifuged at 50 × g for 3 min. After centrifugation, the supernatant was harvested and centrifuged at 800 × g for 5 min. The F4/80+ cells were isolated from the peritoneal, lung, and liver cells using MACS Magnetic Bead columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufac-turer’s instructions (20).

**RNA extraction and real-time PCR analysis**

Total RNA was extracted from the liver and lung tissues or peritoneal cells using Isogen II (Nippon Gene, Tokyo, Japan), and RNA concentration was determined spectrophotometrically at 260 nm. RT-PCR was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The following PCR primers were used: IL-6 sense, 5’-GATACTCAGTCCCAACAGA-3’, and antisense, 5’-GCCATTGCACACCTCTTT-3’; IL-1β sense, 5’-TTTGAGAAGAGCCCATC-3’, and antisense, 5’-TCCAGCAATCCCTTACTG-3’. Nonspecific binding was blocked with 1% BSA in PBS, and the sections were subsequently incubated with rabbit polyclonal anti-KMO Ab (catalog no. 10698-1-AP, RRID: AB_2296744; Proteintech Group) and rat monoclonal anti-F4/80 Abs (catalog no. ab16911, RRID: AB_443548; Abcam) in 1% BSA in PBS overnight at 4°C. Negative controls (without primary Abs) were included for each setup. After primary Ab incubation, the sections were rinsed with PBS and incubated with secondary Abs for 30 min at room temperature. The secondary Abs used were donkey anti-rabbit IgG (H+L) Ab (catalog no. N0104, RRID: AB_623676; R&D Systems, Minneapolis, MI) and goat anti-rat IgG (H+L) Ab (catalog no. ab150157, RRID: AB_272251; Abcam), and nuclei were stained with DAPI (Dojindo Molecular Technologies, Tokyo, Japan). Immunostained slides were observed under a fluorescence microscope BX 51 equipped with a DP74 digital camera (Olympus).
antisense, 5'-TTGGTTGTCATCTCGGAG-3'; TNF-α sense, 5'-TCATGACCACCCATCAAG-3', and antisense, 5'-CAAGAATGGACAT-3'; 18S rRNA sense, 5'-TGCAATCCCAAGCCAACAAG-3', and antisense, 5'-TAAGACGGAGCACTAGCACG-3'; TGF-β sense, 5'-ACAGTCTGCGTACTCTGG-3', and antisense, 5'-CAGGAATGGACAT-3'; and 18S rRNA sense, 5'-GAATTGAGATGGATAGC-3', and antisense, 5'-TATCCGGATTAACAGCACA-3'. The mRNA expression levels of IL-6, IL-1β, TNF-α, IL-10, TGF-β, and 18S rRNA were quantified by quantitative PCR in a 7900HT Fast Real-Time system (Applied Biosystems). PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Data were analyzed using the 7900HT software (version 2.3; Applied Biosystems).

Western blotting and cell extractions

The liver or hepatic macrophages were washed with cold PBS. Nuclear/cytosolic fractions were isolated using the Lysopure Nuclear and Cytoplasmic Extractor Kit (Fujifilm Wako, Osaka, Japan) and the Mitochondria/Cytosol Fractionation kit (catalog no. ab65320; Abcam) according to the manufacturer’s protocol. Ten micrograms of protein was loaded onto 10% Mini-PROTEAN TGX gels (Bio-Rad Laboratories) and transferred to polyvinylidene fluoride membranes. The membranes were first calibrated using the Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories). Protein levels were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD).

Isolation and culture of hepatic macrophages

Hepatic macrophages were isolated as described previously (21) and cultured in high-glucose DMEM supplemented with 10% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin. After stimulation with LPS (100 ng/ml) for the indicated time periods, the cells were fixed with 4% paraformaldehyde/PBS for 10 min and permeabilized with 0.2% Triton X-100/PBS for 5 min. After blocking with 5% BSA/PBS for 30 min, the cells were incubated with primary Abs, namely rat monoclonal anti-F4/80 Ab (catalog no. ab6911, RRID: AB_443548; Abcam) and anti–R–ATF-4 (catalog no. 11815, RRID: AB.2616025; Cell Signaling Technology), in 1% BSA/PBS for 1 h. Then, the cells were washed with PBS and incubated with secondary Abs, donkey anti-rabbit IgG (H+L) Ab (catalog no. NL004, RRID: AB.663767; R&D Systems), and goat anti-rat IgG (H+L) Ab (catalog no. ab150157, RRID: AB.2722518; Abcam) for 1 h. Nuclei were stained with DAPI (Dojin Molecular Technologies). Immunostained slides were observed under a fluorescence microscope BX 51 equipped with a DP74 digital camera (Olympus). At least 200 cells per slide were counted.

For the in vitro assay, hepatic macrophages from KMO<sup>-/-</sup> and KMO<sup>+/+</sup> mice were seeded at a density of 1 x 10<sup>4</sup> per well in a 96-well plate and incubated overnight. After culture, the hepatic macrophages were stimulated with LPS (100 ng/ml), and media supernatants were collected at 0 and 48 h.

Statistical analysis

The experimenters performed the experiments while blinded to the group assignment and evaluation of results. The treatment group sample sizes were designed to provide statistical power while minimizing animal use. All data are expressed as the mean ± SD. The survival rates of the mice were analyzed using the Kaplan–Meier method. Statistical differences between two groups were determined using Student’s t test, and those among more than three groups were determined using one-way ANOVA, followed by a post hoc Tukey test. For the values obtained in the time course experiments, statistical analysis was performed using two-way ANOVA, followed by Bonferroni multiple comparisons test. All comparisons and observations were performed using GraphPad Prism7 (GraphPad Software, San Diego, CA). Statistical significance was set at p < 0.05.

RESULTS

KMO regulates LPS-induced endotoxic shock

<i>S. enterica</i> and<i>E. coli</i> are causative Gram-negative bacteria for sepsis, and their cell wall components, such as LPS, have powerful adjuvant activities that induce the systemic production of inflammatory cytokines (22). To investigate the role of KMO in LPS-induced endotoxic shock, KMO<sup>-/-</sup> and KMO<sup>+/+</sup> mice were injected with<i>S. enterica</i>– and<i>E. coli</i>–derived LPS. KMO<sup>-/-</sup> mice were more sensitive to LPS-induced endotoxic shock and death, whereas KMO<sup>+/+</sup> mice were resistant and survived (Fig. 1A). Interestingly, <i>S. enterica</i>–derived LPS induced septic shock and death to a greater extent than <i>E. coli</i>–derived LPS (Fig. 1A). Similarly, knockout of KMO worsened the survival rate of CLP-induced septic mice. Therefore, we used<i>S. enterica</i>–derived LPS for subsequent experiments. Histological analysis showed that the frequency of mononuclear cells in the livers of KMO<sup>-/-</sup> mice was higher than that of KMO<sup>+/-</sup> mice (Fig. 1B). The serum levels of ALT, a marker of liver damage, and NO were higher in KMO<sup>-/-</sup> mice than those in KMO<sup>+/-</sup> mice 24 h after LPS administration (Fig. 1C, 1D). In contrast, the serum levels of creatinine, a marker of renal damage, and pathological changes in the lungs were not
significantly different between LPS-treated KMO+/− and KMO+/+ mice (data not shown). These results suggest that KMO controls LPS-induced lethal endotoxic shock that likely occurs through liver injury.

**LPS-induced accumulation of F4/80+ cells and elevated KP metabolites in the liver**

To examine the role of hepatic KMO in septic shock, we first compared the KMO activity between the liver and lung tissues. KMO activity was much higher in the liver than in the lung upon LPS administration, and the hepatic expression of KMO was enhanced in a time-dependent manner (Fig. 2A). Consistent with these results, in LPS-treated KMO+ mice, the number of KMO+ cells in the liver was significantly increased compared with that in untreated control mice (p < 0.001; Fig. 2B, 2C). Moreover, upon LPS administration, the number of hepatic F4/80+ cells in KMO+/− mice was higher than that in KMO+/+ mice (Fig. 2D, 2E), implying that LPS-induced accumulation of F4/80+ cells in the liver is regulated by KMO activity. Importantly, KMO was constitutively expressed in hepatic F4/80+ cells (Fig. 2F), and hepatic F4/80− cell expression of KMO was elevated upon administration of LPS (p < 0.05; Supplemental Fig. 1), suggesting that LPS-induced KMO activity regulates the migration of F4/80+ cell precursors (presumably monocytes) into the liver. Some chemokines are critical for the migration of monocytes from the bone marrow and/or peripheral blood into the damaged liver. We examined the hepatic expression of CCL2, CCL5, and CCL25, which are representative chemokines for monocytes, and found that the expression of these chemokines was comparable between septic KMO+/− and KMO−/− mice (Supplemental Fig. 2).

As the expression of KMO was mainly observed in the damaged liver, we next investigated the role of KP metabolites in the liver of LPS-treated mice. In KMO+/− mice, hepatic 3-HK and 3-HAA levels increased 24 h after LPS administration, whereas hepatic KYN, AA, and KA levels did not change significantly (Fig. 2G). In contrast, the levels of KYN, KA, and AA were dramatically elevated in KMO−/− mice. As expected, 3-HK and 3-HAA were not detected in the livers of KMO−/− mice. These results suggest that 3-HK and 3-HAA prevent the development of liver destruction during sepsis.

**Hepatic KMO regulates LPS-induced IL-6 production**

To assess the effects of KMO activity on LPS-induced hepatic inflammation, hepatic cytokine levels were compared between KMO+/− and KMO−/− mice 6 h after LPS administration. The levels of IL-6 and IL-1β mRNA in the liver were significantly higher in KMO−/− mice than those in KMO+/− mice (Fig. 3A). Consistent with these results, serum levels of LPS-induced or CLP-induced IL-6 were significantly higher in KMO−/− mice.
FIGURE 2. Increased hepatic F4/80+ cells in septic KMO−/− mice.

The mice were humanely euthanized at the indicated time points after S. enterica–derived LPS administration (at least n = 5). (A) KMO activity in the liver and lung tissues of KMO+/+ and KMO−/− mice was determined using HPLC. ***p < 0.001, versus KMO+/+ mice on 0 h. (B) Representative images of KMO immunohistochemical staining in livers of KMO+/+ mice and (C) the frequency of KMO-positive cells in the liver. Scale bars, 25 μm. ***p < 0.001, versus 0 h. (D) Representative images of F4/80 immunohistochemical staining in the livers of KMO+/+ and KMO−/− mice 24 h after LPS administration and (E) the frequency of F4/80-positive cells in the liver. Scale bars, 25 μm. Spleen section were used as the positive control. *p < 0.05, ***p < 0.005, versus the indicated time points in KMO−/− mice. (F) Expression of F4/80, KMO, and DAPI nuclei (blue) in the livers of LPS-treated KMO+/+ mice were examined by immunofluorescence staining. The merged image is shown in yellow. Scale bars, 50 μm. Negative controls for F4/80 and KMO were not stained (data not shown). (G) Liver KP metabolite (TRP, KYN, 3-HK, AA, KA, and 3-HAA) levels were determined using HPLC. Data are presented as mean ± SD. *p < 0.05, ***p < 0.001, versus KMO+/+ mice at 0 h; **p < 0.01, ***p < 0.001, versus KMO−/− mice at 0 h. ND, not detected.
than those in KMO+/+ mice (p < 0.001, Fig. 3B). However, serum IL-1β protein levels were not significantly different between these mice (data not shown). Similar results were observed in Ro61-8048–treated KMO+/+ septic mice, which suppressed KMO activity in the liver and serum (Fig. 3C, 3D).

To identify the source of IL-6 in the liver during sepsis, we focused on hepatic macrophages, which are known to produce inflammatory cytokines during sepsis (23). To this end, we isolated F4/80+ cells from the livers of untreated mice as hepatic macrophages and cultured them in the presence or absence of LPS. Upon stimulation with LPS, the production of IL-6 by KMO−/− hepatic macrophages was significantly higher than that by KMO+/+ hepatic macrophages (p < 0.01; Fig. 3E), whereas the expression of IL-6 in HepG2 cells, a human hepatic parenchymal cell line, was not enhanced even after administration of a high dose of LPS (data not shown), implying that the hepatic macrophages are presumably the major source of IL-6 in this model. These results suggest that hepatic KMO-dependent KYN metabolites, such as 3-HK and 3-HAA, are involved in suppressing LPS-induced IL-6 production by hepatic macrophages.

3-HK regulates ATF4 activation

LPS signaling, which is mediated by TLR4, activates several transcription factors, including NF-κB and activating

FIGURE 3. Hepatic KMO regulates IL-6 production in septic mice. (A) The expression levels of IL-6, IL-1β, TNF-α, IL-10, and TGF-β mRNA in the liver were determined using quantitative RT-PCR (RT-qPCR). (B) Serum levels of IL-6 in the KMO+/+ (n = 6) and KMO−/− mice (n = 6) or (C) vehicle and KMO inhibitor (Ro61-8048)–treated mice (n = 9) 6 h after LPS administration or KMO+/+ (n = 13) and KMO−/− mice (n = 13) 6 h after sham or CLP were quantified using an ELISA kit. (D) 3-HK/KYN, indicative of KMO activity, in the liver and serum from vehicle and KMO inhibitor (Ro61-8048)–treated mice were measured using HPLC. (E) Levels of IL-6 produced in the culture media from KMO+/+ and KMO−/− hepatic macrophages by stimulating with LPS (100 ng/ml) at the indicated time points was determined using ELISA. Data are presented as mean ± SD. Statistically significant differences between the groups were determined using two-way ANOVA (A and B) or one-way ANOVA (E), followed by Tukey multiple comparisons test and determined using Student t test (C and D). *p < 0.05, **p < 0.01, ***p < 0.001.
transcription factor 4 (ATF4), which promote the production of inflammatory cytokines such as IL-6 (24, 25). Based on these findings, we examined the activation of NF-κB and ATF4 in the livers of septic mice. LPS-induced nuclear translocation of NF-κB in the liver did not differ between KMO<sup>−/−</sup> and KMO<sup>+/+</sup> mice (Fig. 4A, 4B). In contrast, upon LPS stimulation, the levels of nuclear ATF4 in the liver of KMO<sup>−/−</sup> mice increased significantly compared with those in KMO<sup>+/+</sup> mice (p < 0.05; Fig. 4A, 4B). Along with these changes in signaling pathways, the expression of KMO was also altered (Supplemental Fig. 3).

Hepatic macrophages are involved in the induction of septic shock (26). Therefore, we cultured hepatic macrophages to examine the nuclear translocation of ATF4. LPS-induced nuclear translocation of ATF4 was observed in KMO<sup>−/−</sup> hepatic macrophages, and its translocation was reduced to basal levels in the presence of 3-HK, which is the primary product of KMO (Fig. 4C–E). Collectively, these results suggest that the elevated IL-6 production by KMO<sup>−/−</sup> hepatic macrophages may be induced through the activation of ATF4 and that nuclear translocation may be regulated by 3-HK.

**3-HK improves LPS-induced endotoxic shock**

We investigated the physiological and pharmacological roles of 3-HK in LPS-induced endotoxic shock. At 12 h after LPS administration, KMO<sup>−/−</sup> and KMO<sup>+/+</sup> mice were treated with 3-HK, and the survival rate was monitored for 6 d. As expected, KMO<sup>−/−</sup> mice treated with PBS were highly sensitive to LPS.

**FIGURE 4. KMO regulates the activation of ATF4 in LPS-stimulated hepatic F4/80<sup>+</sup> cells.**

(A and B) Expression levels of ATF4, NF-κB, and lamin B1 nuclear protein and representative images in the liver from KMO<sup>−/−</sup> and KMO<sup>+/+</sup> mice at 0 and 120 min after LPS administration were determined by Western blotting. (C) Representative images of the primary hepatic macrophages stained for F4/80 using immunofluorescence staining. Scale bars, 50 μm. (D) Subcellular localization of ATF4 was compared in primary hepatic macrophages derived from KMO<sup>+/+</sup> mice and from LPS (100 ng/ml) and 3-HK (100 nM)–treated KMO<sup>−/−</sup> mice. Scale bars, 50 μm. (E) Percentage of nuclear translocated cells in which ATF4 merged with DAPI is shown. Data are presented as mean ± SD.

*A p < 0.05, **p < 0.01, using two-way ANOVA followed by Tukey multiple comparisons test.
induced septic shock (Fig. 5A). In contrast, 3-HK–treated KMO<sup>−/−</sup> mice were resistant and survived longer than untreated KMO<sup>−/−</sup> mice. Consistent with this result, serum IL-6 levels after LPS administration were significantly lower in KMO<sup>−/−</sup> mice treated with 3-HK than in KMO<sup>−/−</sup> mice treated with PBS (p < 0.01; Fig. 5B). Similar results were obtained in KMO<sup>+/+</sup> mice (Fig. 5C, 5D). These results clearly show that 3-HK has an inhibitory effect on IL-6 production, which results in the suppression of endotoxic mortality.

**DISCUSSION**

KMO has been considered a therapeutic target for some inflammatory diseases because the application of selective KMO inhibitors can prevent several diseases (27–29). These findings demonstrate the harmful effects of KMO on inflammatory disorders. In this study, we showed that KMO has a beneficial effect on the maintenance of immune homeostasis.

In this study, we found that KMO was predominantly expressed in F4/80<sup>+</sup> cells of the liver, and its expression was enhanced upon LPS administration. Furthermore, the absence of KMO resulted in reduced survival rates and increased number of IL-6–producing F4/80<sup>+</sup> cells in the livers of septic mice, suggesting that hepatic KMO<sup>+</sup>F4/80<sup>+</sup> cells play an important role in immune regulation in this septic model. The liver contains numerous F4/80<sup>+</sup> hepatic macrophages that are highly specialized in phagocytic activity and cytokine production. Hepatic macrophages are divided into three subsets: resident Kupffer cells, macrophages, and monocyte-derived macrophages (30, 31). However, it is currently difficult to distinguish monocyte-derived hepatic macrophages from resident Kupffer cells and hepatic macrophages based on cell surface markers, localization, and function. In this context, it is possible to distinguish between recruited and resident cells at early time points after stimulation. Upon LPS stimulation, monocytes that migrated into the liver did not differentiate into hepatic macrophages within 24 h (32), and F4/80<sup>+</sup> hepatic macrophages that expressed KMO within 6 h after the stimulation appeared to be a subset of the resident cells. Notably, we also found that at late time points after LPS stimulation, the frequency of F4/80<sup>+</sup> cells in the liver was higher in KMO<sup>−/−</sup> mice than in KMO<sup>+/+</sup> mice, suggesting that the expression of chemokines in monocytes is enhanced in the liver of KMO<sup>−/−</sup> mice. However, we did not observe any differences in the hepatic expression of chemokines. In this respect, KYN is known to enhance CCL2–induced migration of monocytes (33), implying that enhanced monocyte migration is dependent on the KYN-sufficient environment in the liver of KMO<sup>−/−</sup> mice. Interestingly, monocyte-derived hepatic macrophages are recruited to the damaged liver to promote hepatic regeneration (34). Therefore, KMO<sup>+</sup> hepatic macrophage–derived KP metabolites have host-protective roles and prevent hepatic diseases.

IL-6 is involved in inflammatory responses, and the inhibition of IL-6 activity is effective in treating bacterial sepsis (35, 36). In contrast, LPS-induced mortality is not affected regardless of the

**FIGURE 5. 3-HK enhances resistance to LPS-induced endotoxemia.**

KMO<sup>−/−</sup> (n = 6) and KMO<sup>+/+</sup> mice (n = 10) were treated with LPS from *S. enterica*. 3-HK or PBS was administered to both groups every 12 h after LPS injection. (A and C) Survival rates of mice from 0 to 6 d postinjection. Statistically significant differences between the groups were determined using the log-rank test. (B and D) Serum levels of IL-6 in 3-HK– or PBS-treated KMO<sup>−/−</sup> and KMO<sup>+/+</sup> mice 6 h after LPS injection were quantified using ELISA. Data are presented as mean ± SD. **p < 0.01, using Student t test.
presence or absence of IL-6 (37), indicating that the physiological role of IL-6 differs between sepsis models. Because we applied an LPS-induced sepsis model in this study, the elevated mortality observed in KMO−/− mice appears to be independent of IL-6. Reportedly, excessive amounts of LPS-induced IL-6 inhibit liver regeneration after partial hepatectomy (38). Supporting this finding, we observed severe hepatocyte death in septic KMO−/− mice, which correlates with excessive IL-6 levels, suggesting that IL-6 is an initiator for hepatic diseases in sepsis.

We also found that the liver is the major source of IL-6 in the sera of septic KMO−/− mice and that the hepatic production of IL-6 is largely dependent upon macrophages. However, besides macrophages, stromal cells including fibroblasts and endothelial cells also have the ability to produce IL-6 during sepsis (39). Therefore, we cannot rule out the possibility that KMO−/− stromal cells also contribute to elevated serum IL-6 levels and participate in hepatic diseases. Our findings raise the question of whether KMO regulates IL-6 production. We found that elevated IL-6 production was induced in LPS-treated KMO−/− mice but was inhibited by treatment with 3-HK, which is consistent with the finding that KMO+/+ mice are resistant to septic shock. Although 3-HK treatment was presumed to be more effective in KMO−/− mice than in KMO+/+ mice, the opposite was observed. In KMO+/+ mice, 3-HK is normally produced upon LPS administration, indicating that the dose of 3-HK administered was excessive. However, we were able to confirm the role of 3-HK as an anti-inflammatory molecule in this study.

LPS-induced inflammatory cytokine production is generally promoted by NF-κB and ATF4 signaling (24, 25, 40, 41). Interestingly, NF-κB activation is inhibited by 3-HAA, a KP metabolite (42). These reports suggest that KP metabolites regulate LPS-induced inflammatory cytokine production via the nuclear transcription pathway. We showed that LPS-induced nuclear translocation of ATF4 in hepatic macrophages was inhibited in the presence of 3-HK. LPS-induced IL-6 production mediated by ATF4 is associated with mitochondrial stress (43). Importantly, KMO is predominantly localized in the outer membrane of mitochondria (6), implying that LPS-induced IL-6 production is involved in mitochondrial dysfunction. The underlying mechanism by which 3-HK regulates ATF4 activation remains elusive. Oxidative stress promotes the nuclear translocation of ATF4, which is inhibited by antioxidants (44). Oxidative stress induced by mitochondrial reactive oxygen species is also associated with LPS-induced sepsis (45). Interestingly, 3-HK can function as an endogenous antioxidant (46, 47), suggesting that 3-HK prevents the nuclear translocation of ATF4 mediated by LPS-induced oxidative stress, thereby preventing sepsis. Therefore, our findings may explain the beneficial effects of KP metabolites on septic shock. However, we could not elucidate the molecular mechanism by which 3-HK prevents ATF-4 signaling. Further studies are needed to clarify the underlying mechanism.

Sepsis is a common feature of bacterial infection. Antibiotic treatment is widely accepted as the first-line therapy for sepsis. However, excessive antibiotic treatment causes the emergence of multidrug-resistant bacteria in patients. In this context, in addition to antibiotic treatments, immunotherapy has shed light on the treatment of sepsis. Indeed, PD-1, which was originally identified as a target molecule for cancer immunotherapy, may be a potential therapeutic target for designing measures to modulate host immune responses, thereby preventing the detrimental effects of sepsis (48). In this study, we showed that 3-HK, which regulates IL-6 production, is a potential candidate molecule for the development of immunotherapy for sepsis. Collectively, a combination of antibiotic treatments and immunotherapy appears to be important for the treatment of sepsis.

In conclusion, our results suggest that the inclusion of 3-HK as part of the treatment approach for sepsis or as an adjuvant therapy might reduce the overproduction of IL-6 responsible for severe inflammatory diseases and ultimately improve the survival rates of patients with sepsis.

DISCLOSURES

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