Molecules and Cells

Contributory Role of BLT2 in the Production of Proinflammatory Cytokines in Cecal Ligation and Puncture-Induced Sepsis

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BLT2 is a low-affinity receptor for leukotriene B_4, a potent lipid mediator of inflammation generated from arachidonic acid via the 5-lipoxygenase pathway. The aim of this study was to investigate whether BLT2 plays any role in sepsis, a systemic inflammatory response syndrome caused by infection. A murine model of cecal ligation and puncture (CLP)-induced sepsis was used to evaluate the role of BLT2 in septic inflammation. In the present study, we observed that the levels of ligands for BLT2 (LTB_4 [leukotriene B_4] and 12(*S*)-HETE [12(*S*)-hydroxyeicosatetraenoic acid]) were significantly increased in the peritoneal lavage fluid and serum from mice with CLP-induced sepsis. We also observed that the levels of BLT2 as well as 5-lipoxygenase (5-LO) and 12-LO, which are synthesizing enzymes for LTB_4 and 12(*S*)-HETE, were significantly increased in lung and liver tissues in the CLP mouse model. Blockade of BLT2 markedly suppressed the production of sepsis-associated cytokines (IL-6 [interleukin-6], TNF-α [tumor necrosis factor alpha], and IL-1β [interleukin-1β] as well as IL-17 [interleukin-17]) and alleviated lung inflammation in the CLP group. Taken together, our results suggest that BLT2 cascade contributes to lung inflammation in CLP-induced sepsis by mediating the production of inflammatory cytokines. These findings suggest that BLT2 may be a potential therapeutic target for sepsis patients.

Keywords: BLT2, cecal ligation and puncture, cytokines, leukotrienes, sepsis

INTRODUCTION

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection and is the most frequent cause of mortality in intensive care units (Salomao et al., 2019; Singer et al., 2016). Until recently, the incidence of sepsis has been increasing, and provisional extrapolation from meta-analyses has suggested that sepsis may cause or contribute to up to 5.3 million deaths worldwide per annum (Fleischmann et al., 2016; Vakkalanka et al., 2018). Many previous studies have shown that hyperinflammation is closely associated with multiple organ dysfunction syndrome in sepsis (Cho et al., 2020; Choi et al., 2020; Delano and Ward, 2016; Salomao et al., 2019; van der Poll et al., 2017; Ziesmann and Marshall, 2018). Advances in understanding sepsis have revealed that the "cytokine storm" during sepsis contributes to cell or organ damage (Chousterman et al., 2017). Systemic activation of the innate immune system by infection results in a severe and persistent inflammatory response characterized by an excessive release of proinflammatory cyto-

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kines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-17 (IL-17) (Delano and Ward, 2016; Rittirsch et al., 2008). Proinflammatory cytokines in sepsis stimulate the production of acute phase proteins and induce immune cell infiltration at the infection site (Chaudhry et al., 2013; Li et al., 2012; Molano Franco et al., 2019). The release of these cytokines will induce the production and release of new cytokines that will in turn cause cell and organ damage (Chousterman et al., 2017; Delano and Ward, 2016).

Leukotriene B_4 (LTB_4) is one of the major inflammatory lipid mediators derived via the arachidonic acid pathway, and it works as a chemoattractant molecule for leukocytes such as neutrophils (Yokomizo et al., 2001). This proinflammatory molecule has two receptors, BLT1 and BLT2. These receptors are members of the G protein-coupled receptor (GPCR) family and are expressed on the surface of cells (Jang et al., 2017; Lundeen et al., 2006; Tager and Luster, 2003). BLT1, a high-affinity receptor of LTB_4, is normally expressed on the surface of inflammatory immune cells such as macrophages and neutrophils. On the other hand, BLT2 has a low-affinity function for LTB_4, and it is expressed in various tissues, such as the lung and liver (Kim et al., 2009; Tager and Luster, 2003). Our previous research showed that LTB_4 receptors play critical mediatory roles in the development of LPS-induced endotoxic shock (Kwon et al., 2019). Other previous experimental results have indicated that LTB_4 signaling contributes to hyperinflammation in a sepsis model (Benjamim et al., 2005; Matsukawa et al., 1999; Rios-Santos et al., 2003). In the cecal ligation and puncture (CLP)-induced sepsis model, LTB_4 augments cytokine production, neutrophil migration, and organ dysfunction (Bitto et al., 2012; Liu et al., 2015; Monteiro et al., 2014). Though it is suggested that BLT1 is associated with CLP-induced septic inflammation (Li et al., 2015; Scott et al., 2004), the role of BLT2 in the CLP model has not been previously studied.

In the current study, we investigated the mediatory role of BLT2 in the synthesis of proinflammatory cytokines in the CLP-induced sepsis mouse model. Blockade of BLT2 decreased the levels of IL-6, IL-17, IL-1β and TNF-α production in the serum and peritoneal lavage fluid (PF). Furthermore, we found that the inhibition of BLT2 reduced lung tissue damage and ameliorated the survival rate. Consequently, these results indicate that BLT2 plays an important mediatory role in the development of the pathological condition of sepsis. To our knowledge, this report is the first to define the role of BLT2 in CLP-induced sepsis. Our findings may facilitate the development of therapeutic strategies against sepsis, one of the life-threatening diseases.

MATERIALS AND METHODS

Reagents

Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (USA). U75302 was obtained from Enzo Life Sciences (USA). LY255283 was obtained from Cayman Chemical (USA).

Cecal ligation and puncture mouse model

Male C57BL/6 mice (8 to 9 weeks old) were obtained from Orient Bio (Korea). CLP was performed as previously described (Rittirsch et al., 2009). Briefly, mice were anesthetized with isoflurane, and the mouse cecum was fully exposed through an incision on the ventral surface of the abdomen. The cecum was ligated at 70% of its total length with 4-0 silk and then a through-and-through puncture was made with a 22-gauge needle (BD Biosciences, USA). Sham-operated mice underwent the same surgery without ligation and puncture of the cecum. In all groups of mice, the incision was closed by suturing with 4-0 silk. The doses of LY255283 (10 mg/kg) and U75302 (0.5 mg/kg) were chosen based on previous reports (Kwon et al., 2019), and under these doses, we did not observe any toxicity or off-target effects. To investigate the specific contributory roles of BLT1/2 in the production of proinflammatory cytokines and lipid mediators during CLP-induced septic pathogenesis, we chose to inject the tested drugs at 1 h and 12 h after CLP surgery in the CLP-induced model. Mice were maintained in a temperature-controlled facility under a 12 h light-dark cycle with free access to water and food. All experimental animals used in this study were treated according to ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments) approved by the Institutional Animal Care and Use Committee of Korea University (KU-IACUC). The experimental protocols were approved by KU-IACUC (approval No. KU-IACUC-2019-0056) and performed in accordance with relevant guidelines and regulations.

Preparation of tissue lysates and immunoblot analysis

The experiment was performed as described previously (Kwon et al., 2019; Lee et al., 2015). Lung and liver tissues were homogenized and lysed in lysis buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.02% NaN_3, 100 mM phenylmethyl-sulfonyl fluoride, 1 M sodium fluoride, leupeptin [0.5 mg/ml], aprotinin [1 mg/ml], and pepstatin A [5 mg/ml]) at 4°C. The lysate protein samples were heated at 95°C for 5 min and subjected to SDS-PAGE. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane for 1 h at 100 V. The membrane was incubated for 1 h with TBS-T containing 5% nonfat dried milk at room temperature (RT) and for 1 h at RT with primary antibodies at a 1:1,000 dilution (1:500 for BLT1 and BLT2 or 1:2,000 for β-actin) in TBS-T. The membrane was then incubated for 1 h at RT with HRP-conjugated secondary antibodies before the detection of immune complexes with an enhanced chemiluminescence kit (Amersham Biosciences, UK). Antibody against 5-LO was obtained from BD biosciences, antibody against 12-LO were obtained from Santa Cruz Biotechnology (USA), antibodies against BLT1 and BLT2 were obtained from Enzo Life Sciences, and antibodies against p-IκBα and β-actin (loading control) were obtained from Cell Signaling Technology (USA). Size estimates for proteins were obtained using molecular weight standards from Thermo Fisher Scientific (USA).

Measurement of IL-6, TNF-α, IL-1β, IL-17, LTB4 and 12(S)-HETE

The levels of IL-6, TNF-α, IL-1β, IL-17, LTB_4 or 12(*S*)-HETE were quantified in the supernatants of the PF or serum using

an ELISA kit (R&D Systems [USA] for IL-6, TNF-α, IL-1β, and IL-17; Enzo Life Sciences for LTB_4 and 12(*S*)-HETE) according to the manufacturer's instructions. PF was obtained from mice by peritoneal washing using 2 ml of PBS, and the collected PF was centrifuged at 1,000 × *g* for 3 min. Then, the supernatant was collected for ELISA.

Analysis of lung and liver histology

Lung and liver tissues were harvested, fixed in 10% formaldehyde for 3 weeks and embedded in paraffin. Lung and liver sections (5 μm thickness) were mounted onto Superfrost Plus glass slides (Thermo Fisher Scientific), deparaffinized and stained with H&E. All images were acquired using a BX51 microscope (Olympus, Japan) equipped with a DP71 digital camera (Olympus). A quantitative histological analysis was performed to measure the degree of inflammation by six independent, blinded investigators as previously described (Li et al., 2013; Siegmund et al., 2002).

Survival studies

For survival studies, we tested the effect of the BLT2 antagonist in a mild-moderate sepsis condition. For this condition, the cecum was ligated at 50%, not 70%, of its total length. In addition, the inhibitors were injected intraperitoneally at 1 h before and 12 h after CLP surgery and monitored for survival up to 6 days.

Statistical analysis

Statistical analyses of ELISA results were performed with oneway ANOVA, followed by Bonferroni's post hoc test. Comparisons between tissue histopathology scores were analyzed by one-way ANOVA followed by Tukey's post hoc test. Survival analysis was performed with Kaplan-Meier curves and logrank test. IBM SPSS Statistics for Windows (ver. 25.0; IBM, USA) and GraphPad Prism 5.0 (GraphPad Software, USA) were used for the statistical analysis. The results are presented as the mean ± SD. *P* values < 0.05 indicated statistical significance.

RESULTS

The levels of BLT2 and its ligands are highly elevated in CLP-induced sepsis

To investigate the role of BLT2 in the CLP-induced sepsis mouse model, we analyzed the expression level of BLT2 in lung and liver tissues. As shown in Fig. 1A, the levels of BLT2 were increased in lung and liver tissues in a time-dependent manner after CLP surgery (Fig. 1A). In addition, the levels of 5-LO and 12-LO, which catalyze the synthesis of LTB_4 and 12(*S*)-HETE from arachidonic acid, were highly increased in lung and liver tissues (Fig. 1A). The level of BLT1 was also increased in the lung and liver (Fig. 1A). Next, we measured the levels of BLT2 ligands (LTB_4 and 12(*S*)-HETE) in the serum and PF. At 6 h or 18 h after CLP surgery, the levels of LTB_4 and 12(*S*)-HETE were markedly elevated in both the serum and PF (Figs. 1B and 1C). Together, these results indicate that the levels of BLT2 and its ligands are highly enhanced in CLP-induced sepsis.

BLT2 contributes to the synthesis of sepsis-associated inflammatory cytokines in CLP-induced sepsis

To examine the role of BLT2 in the production of sepsis-associated cytokines in the CLP-induced mouse model, we intraperitoneally injected LY255283, an inhibitor of BLT2, at 1 h and 12 h after CLP surgery. The quantification of cytokines through ELISA showed that treatment with LY255283

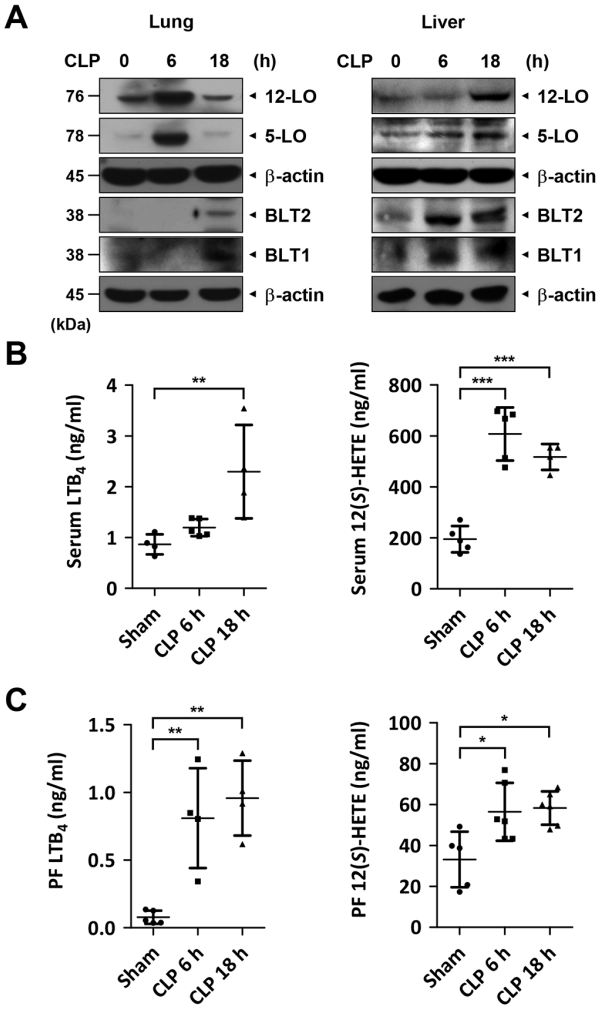

Fig. 1. The levels of BLT2 and its ligands are highly elevated in CLP-induced sepsis. Mice were randomly divided into three groups, underwent CLP or sham surgery, and sacrificed at the indicated time points. Lung and liver tissues were harvested for immunoblot assays, and the PF and serum were collected for ELISAs. (A) Mouse lung and liver tissues were homogenized, and the protein levels of 5/12-LO, BLT1 and BLT2 were analyzed by immunoblot assays. Data are representative of three independent experiments with similar results. (B) The levels of LTB_4 and 12(*S*)-HETE in the serum were analyzed using ELISA after CLP. (C) The levels of LTB_4 and 12(*S*)-HETE in the PF were analyzed using ELISA after CLP. Data are shown as the mean ± SD (n = 4-6 per group). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus the control group.

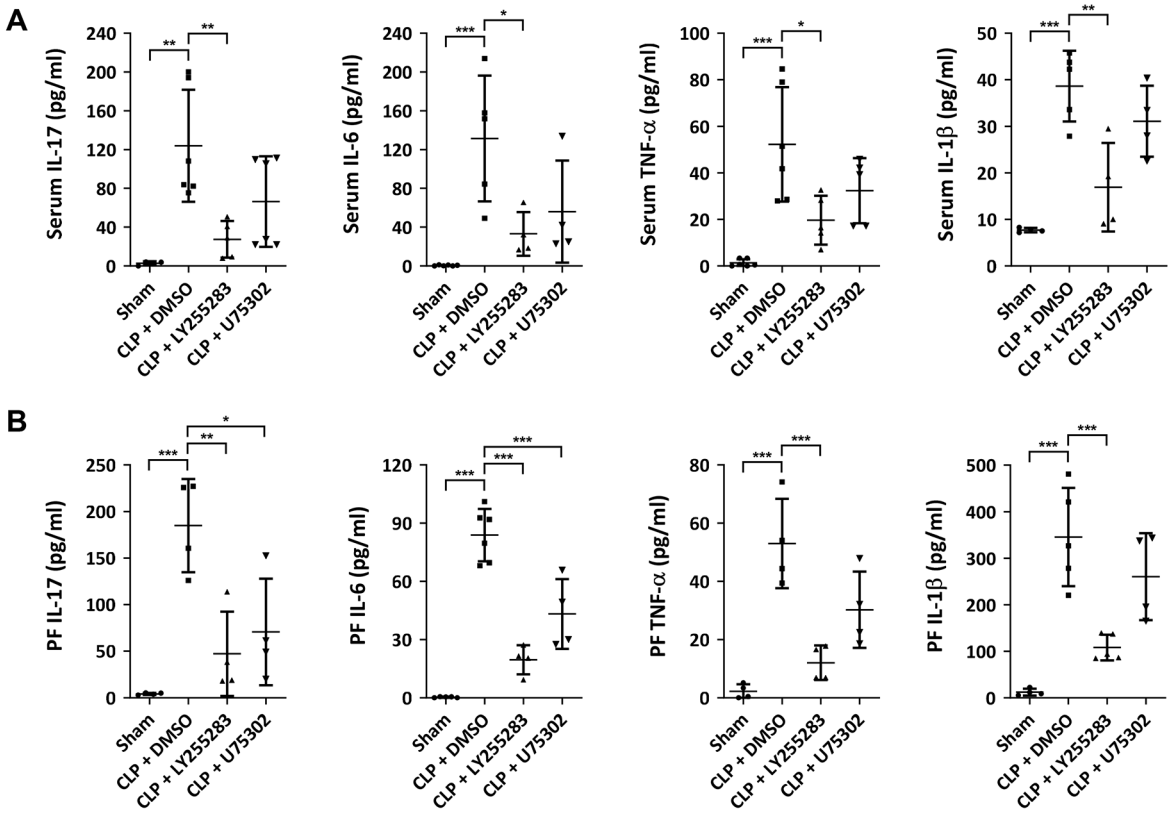

Fig. 2. BLT2 is critical for the synthesis of sepsis-associated inflammatory cytokines in CLP-induced sepsis. Mice were intraperitoneally administered U75302 (0.5 mg/kg) or LY255283 (10 mg/kg) at 1 h and 12 h after the CLP procedure, and blood and PF were collected 18 h after CLP. (A) The levels of IL-17, IL-6, TNF-α, and IL-1β in the serum were analyzed using ELISAs. (B) The levels of IL-17, IL-6, TNF-α, and IL-1β in the PF were analyzed using ELISAs. Data are shown as the mean ± SD (n = 4-6 per group). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus each control group.

suppressed the levels of IL-17, IL-6, IL-1β, and TNFα in the serum and PF (Fig. 2). Similarly, treatment with U75302, an inhibitor of BLT1, had partial suppressive effect on the levels of these cytokines in serum and PF (Fig. 2). Together, these results suggest that BLT2 and possibly BLT1 contribute to the synthesis of sepsis-associated cytokines in the CLP-induced sepsis model.

BLT2 regulates NF-κB activation in the lungs of mice with CLP-induced sepsis

Previous studies have suggested that NF-κB activation is essential for the development of sepsis (Bitto et al., 2012; Crooks and Stockley, 1998; Saeki and Yokomizo, 2017; Serezani et al., 2011). Thus, we investigated whether BLT2 plays any role in NF-κB activation in CLP-induced sepsis. Immunoblot results clearly showed that LY255283 treatment reduced the levels of p-IκBα in lung tissue but not in liver tissue (Fig. 3A). Interestingly, U75302 treatment reduced the p-IκBα levels in liver tissue but not in lung tissue (Fig. 3B). These results suggest that BLT1 and BLT2 may contribute to CLP-induced inflammation in a tissue-specific manner.

Lung inflammation is suppressed by BLT2 blockade in CLP-induced sepsis

To examine whether BLT2 contributes to inflammation in the sepsis model, sections of lung and liver tissues were prepared. Histopathological analysis with H&E staining showed increased lung (Fig. 4A) and liver (Fig. 4B) tissue inflammation by CLP-induced sepsis. Alveolar hemorrhage and the influx of immune cells in lung tissue were markedly suppressed by LY255283 (Fig. 4A). On the other hand, BLT1 inhibition by U75302 treatment attenuated necrosis and leukocyte infiltration into the parenchyma in liver tissue (Fig. 4B). These results were verified by a quantitative analysis of inflammation scores (Figs. 4A and 4B). Together, these results show that BLT2 is more closely associated with lung inflammation in CLP-induced sepsis, while BLT1 is associated with liver inflammation, in parallel with the results presented in Fig. 3.

DISCUSSION

In the present study, we found that the levels of BLT2 and its ligands are highly elevated in CLP-induced sepsis. Additionally, we found that BLT2 mediates IL-6, TNF-α, IL-1β, and IL-17 production in the serum and PF in CLP-induced sepsis. Furthermore, BLT2 inhibition suppressed NF-κB activation and

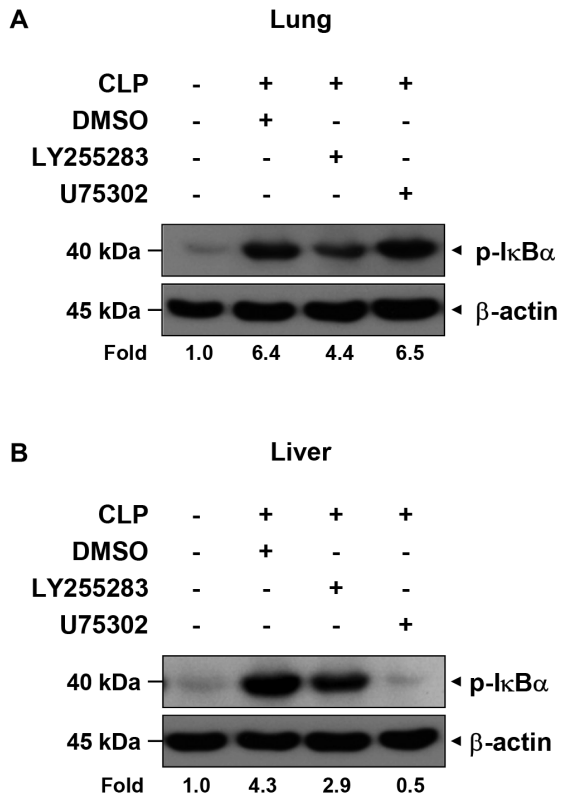

Fig. 3. BLT2 regulates NF-κB activation in the lungs of mice with CLP-induced sepsis. Mice were intraperitoneally injected with U75302 (0.5 mg/kg) or LY255283 (10 mg/kg) at 1 h and 12 h after CLP surgery. Lung and liver tissues were harvested 18 h after CLP and homogenized for immunoblot assays. (A) The protein levels of p-IκBα in lung tissue were analyzed by immunoblot assays. (B) The protein levels of p-IκBα in liver tissue were analyzed by immunoblot assays. Data are representative of three independent experiments with similar results. The intensities were quantified using ImageJ program and are expressed as the fold change.

inflammation in the lung tissues of mice with CLP-induced sepsis, together suggesting that BLT2 contributes to lung inflammation in CLP-induced sepsis by producing inflammatory cytokines.

CLP-induced sepsis was previously shown to be closely associated with the enhanced production of inflammatory cytokines (Chousterman et al., 2017). The critical roles of IL-6, TNF-α, and IL-1β in the CLP-induced sepsis mouse model are already known (Dejager et al., 2011; Matsukawa et al., 1999; Molano Franco et al., 2019; Riedemann et al., 2003), and IL-17 was recently reported to be involved in polymicrobial infection (Li et al., 2012; Luo et al., 2016). These cytokines have been reported to induce neutrophil influx, tissue injury, and hypotension in sepsis (Rittirsch et al., 2008). Despite the reported roles of IL-6, TNF-α, IL-1β, and IL-17 in the development of CLP-induced sepsis, the detailed signaling mechanisms underlying their production remain incompletely elucidated. In the present study, the inhibition of BLT2 significantly reduced IL-6, TNF-α, IL-1β, and IL-17 production in the PF and serum in the CLP-induced sepsis mouse model (Fig. 2). Therefore, we propose that BLT2 contributes to the production of systemic inflammatory cytokines in CLP-induced sepsis. Still, we cannot exclude the contribution of BLT1 to the cytokine production in the CLP-induced septic inflammation (Fig. 2). Indeed, previous studies suggested that BLT1 is associated with neutrophil influx in peritoneum and organ injury in CLP-induced sepsis (Li et al., 2015; Scott et al., 2004). In agreement with our results, a previous report showed that 5-LO, the enzyme that catalyzes the production of LTB_4, is associated with lung injury in a CLP model (Monteiro et al., 2014). LTB_4, a product of 5-LO, has been shown to be increased in the PF, lung, and liver after CLP, suggesting that it is also associated with sepsis (Li et al., 2015; Matsukawa et al., 1999; Monteiro et al., 2014; Rios-Santos et al., 2003). In addition, a previous report showed that baicalein has an inhibitory effect on CLP-induced liver injury (Liu et al., 2015), supporting a potential role for 12-LO in CLP-induced sepsis.

NF-κB activation is essential for the development of sepsis (Bitto et al., 2012; Crooks and Stockley, 1998; Saeki and Yokomizo, 2017; Serezani et al., 2011). In agreement with the proposed role of BLT2 in sepsis, we found that the inhibition of BLT2 significantly reduced IκBα phosphorylation in lung tissue (Fig. 3). Interestingly, the inhibition of BLT1 was shown to reduce IκBα phosphorylation in liver tissue after CLP surgery (Fig. 3). Histopathological analysis of lung and liver tissues showed similar results in parallel with the results presented in Fig. 3. Thus, we suspect that the BLT1- and BLT2-linked cascades may regulate inflammation in a tissue-specific manner in the CLP-induced mouse model. The reason for these phenomena is not clear at the moment, but it may be due to different expression patterns of these receptors; BLT1 is normally expressed on the surface of inflammatory immune cells, and BLT2 is expressed relatively ubiquitously (Kim et al., 2009; Saeki and Yokomizo, 2017; Tager and Luster, 2003). Clearly, further studies are needed to elucidate the detailed roles of BLT1 and BLT2 in tissue inflammation in CLP-induced sepsis.

Additionally, we investigated the effect of LY255283 and U75302 on the survival of mice with CLP-induced sepsis. For survival studies, we tested the effect of the BLT2 antagonist in a less severe sepsis condition since we could not detect any beneficial effect in our established CLP-induced severe sepsis model (data not shown). To induce a less severe sepsis condition, the cecum was ligated at 50%, not 70%, of its total length. In addition, the inhibitors were injected intraperitoneally at 1 h before and 12 h after CLP surgery. Cleary, we could observe that BLT2 antagonist administration ameliorates the survival rate (Fig. 4C), further supporting the suggested contributory role of BLT2 in CLP-induced sepsis. In summary, our results demonstrate that CLP-induced sepsis stimulates BLT2 signaling, which mediates the synthesis of the cytokines IL-6, TNF-α, IL-1β, and IL-17. We also observed that BLT2 contributes to lung inflammation via NF-κB activation in CLP-induced sepsis. Furthermore, BLT2 antagonist administration improved survival in CLP-induced sepsis. These findings suggest that BLT2 may be a novel target for the treatment of inflammatory complications in sepsis patients.

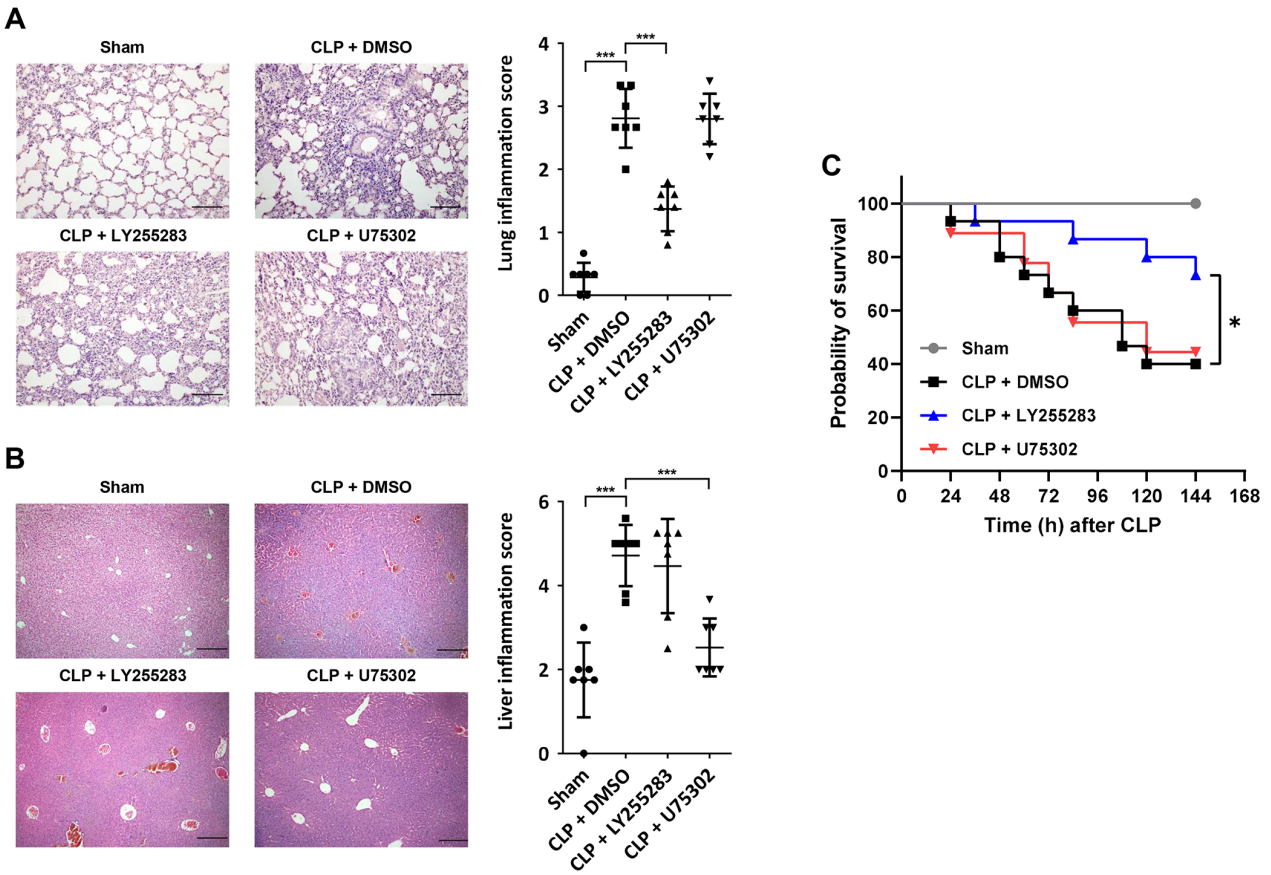

Fig. 4. Lung inflammation is suppressed by BLT2 blockade in CLP-induced sepsis. (A and B) Mice were intraperitoneally injected with U75302 or LY255283 at 1 h and 12 h after CLP surgery. Lung and liver tissues were harvested 18 h after CLP, fixed in formalin and embedded in paraffin for section staining. (A) Lung tissue sections from the indicated groups were stained with H&E. Peribronchial and perivascular lung inflammation was measured and scored. Data are shown as the mean ± SD (n = 7 per group). ***$P < 0.001$ versus each control group. Scale bars = 100 μm. (B) Liver tissue sections from the indicated groups were stained with H&E. Necrosis and leukocyte infiltration into the parenchyma were measured and scored. Data are shown as the mean ± SD (n = 7 per group). ***$P < 0.001$ versus each control group. Scale bars = 200 μm. (C) Survival rate of mice after CLP surgery. Mice were intraperitoneally injected with U75302 or LY255283 at 1 h before and 12 h after CLP surgery and monitored for survival up to 144 h (n = 8 for sham; n = 15 for CLP + DMSO and CLP + LY255283; n = 9 for CLP + U75302).

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AUTHOR CONTRIBUTIONS
D.P. and M.R. planned the study, performed the experiments, analyzed the data, and wrote the manuscript. A.J.L. and D.W.K. performed the experiments. Y.C. advised the statistical analysis and analyzed the data. J.H.K. supervised the study and wrote the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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