ORIGINAL RESEARCH

Pivotal Role of the 5-Lipoxygenase Pathway in Lung Injury after Experimental Sepsis

Ana Paula T. Monteiro[1], Erico Soledade[2], Carla S. Pinheiro[1], Ludmilla Dellatorre-Teixeira[1], Gisele P. Oliveira[1], Mariana G. Oliveira[1], Marc Peters-Golden[3], Patricia R. M. Rocco[1], Claudia F. Benjamim[2]*, and Claudio Canetti[1]*

[1]Instituto de Biofísica Carlos Chagas Filho and [2]Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; and [3]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan

Abstract

Postsepsis lung injury is a common clinical problem associated with significant morbidity and mortality. Leukotrienes (LTs) are important lipid mediators of infection and inflammation derived from the 5-lipoxygenase (5-LO) metabolism of arachidonate with the potential to contribute to lung damage after sepsis. To test the hypothesis that LTs are mediators of lung injury after sepsis, we assessed lung structure, inflammatory mediators, and mechanical changes after cecal ligation and puncture surgery in wild-type (WT) and 5-LO knockout ($5\text{-LO}^{-/-}$) mice and in WT mice treated with a pharmacologic LT synthesis inhibitor (MK886) and LT receptor antagonists (CP105,696 and montelukast). Sixteen hours after surgery, WT animals exhibited severe lung injury (by histological analysis), substantial mechanical impairment (i.e., an increase in static lung elastance), an increase in neutrophil infiltration, and high levels of LTB_4, cysteinyl-LTs (cys-LTs), prostaglandin E_2, IL-1β, IL-6, IL-10, IL-17, KC (CXCL1), and monocyte chemotactic protein–1 (CCL2) in lung tissue and plasma. $5\text{-LO}^{-/-}$ mice and WT mice treated with a pharmacologic 5-LO inhibitor were significantly protected from lung inflammation and injury. Selective antagonists for BLT1 or cys-LT1, the high-affinity receptors for LTB_4 and cys-LTs, respectively, were insufficient to provide protection when used alone. These results point to an important role for 5-LO products in sepsis-induced lung injury and suggest that the use of 5-LO inhibitors may be of therapeutic benefit clinically.

Keywords: 5-lipoxygenase; cytokines; leukotrienes; lung injury; sepsis

Clinical Relevance

Acute lung injury after sepsis is a common clinical problem associated with significant morbidity and mortality. Here we provide evidence that 5-lipoxygenase products are involved in the injury observed, and we suggest that 5-lipoxygenase inhibition could have beneficial effects when its use is carefully considered.

Sepsis leading to shock and organ failure is associated with high rates of mortality and morbidity and enormous health care costs (1). Sepsis is the third leading cause of death and is responsible for almost 10% of deaths in the United States (2). Management of patients with sepsis is largely limited to supportive therapies, reflecting an incomplete understanding of the underlying pathophysiology. Intestinal perforation and contamination of the abdominal cavity with microbes and toxins stimulate local formation of numerous proinflammatory compounds, which subsequently diffuse into the systemic circulation (3). The lung is the most sensitive and clinically important end organ for the systemic inflammatory response in abdominal sepsis (4).

Leukotrienes (LTs) are lipid mediators produced from the 5-lipoxygenase (5-LO) metabolism of arachidonic acid. The 5-LO

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*These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Claudio Canetti, Ph.D., Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Centro de Ciências e Saúde (CCS), Av. Carlos Chagas Filho 373, Bloco C sala 24, Ilha do Fundão, Rio de Janeiro, RJ, Brazil, 22541-900. E-mail: ccanetti@biof.ufrj.br

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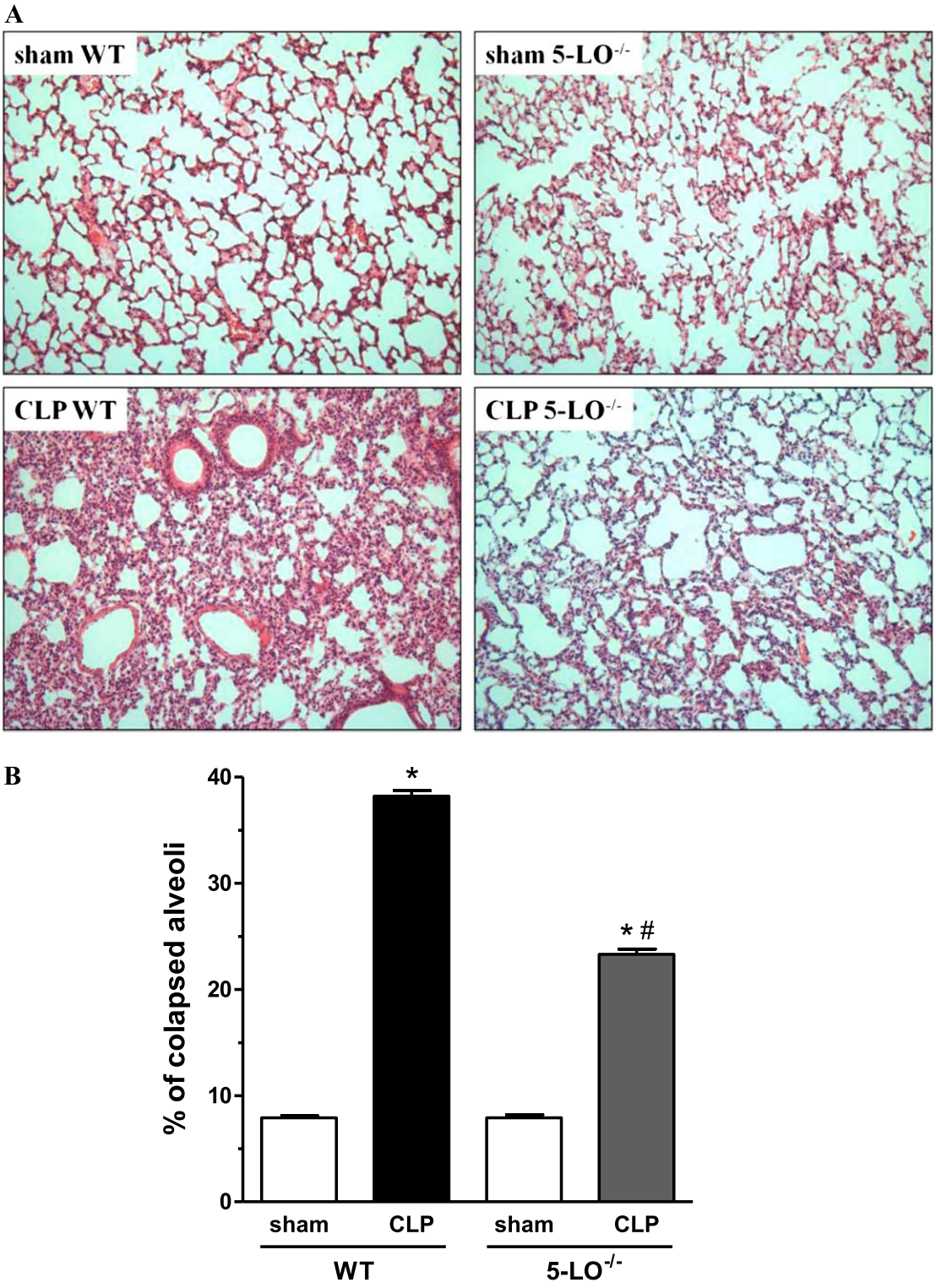

Figure 1. Morphologic changes in sham and cecal ligation and puncture (CLP) wild-type (WT) (sv129 strain) and 5-lipoxygenase knockout (5-LO$^{-/-}$) mice. (*A*) Representative lung sections from sham/WT (*upper left*), CLP/WT (*lower left*), sham/5-LO$^{-/-}$ (*upper right*), and CLP/5-LO$^{-/-}$ mice (*lower right*) obtained 16 hours after surgery and stained with hematoxylin and eosin. (*B*) Morphometry of sham and CLP WT and 5-LO$^{-/-}$ mice. Lungs were collected 16 hours after surgery and prepared for histology. Data are presented as the mean ± SEM. *$P < 0.05$ compared with sham group. #$P < 0.05$ compared with CLP WT mice.

enzyme, together with 5-LO activating protein (FLAP), oxygenates the C5 carbon of arachidonic acid to yield LTA_4. This unstable intermediate can be hydrolyzed to LTB_4, a potent leukocyte chemoattractant and activator, or can be conjugated with glutathione to form LTC_4, which is then metabolized extracellularly to LTD_4 and LTE_4. Collectively known as cysteinyl-LTs (cys-LTs), these are important inducers of smooth muscle contraction and vascular permeability (5). Both classes of LTs play important roles in host defense against a variety of infectious agents, in part by enhancing phagocytosis and the killing capacity of leukocytes (6). We have previously reported that 5-LO knockout ($5\text{-LO}^{-/-}$) mice exhibit an impaired clearance and higher susceptibility to intrapulmonary *Klebsiella pneumonia* challenge (7). In a cecal ligation and puncture (CLP) model of peritonitis with severe sepsis, $5\text{-LO}^{-/-}$ mice showed a decrease in peritoneal neutrophil recruitment and an increase in the number of bacteria recovered from the peritoneal cavity. Despite this impairment of local innate immunity, the null mice exhibited a marked improvement in survival. This protection was also seen in wild-type (WT) animals treated with the LT synthesis inhibitor MK886 (8).

Several reports in the literature suggest that the 5-LO pathway is important in the development of lung injury induced by hemorrhagic shock, hyperoxia, LPS, mechanical ventilation, and ischemia-reperfusion (9–13); however, no information is available regarding its role in microbial sepsis-induced lung injury. In the current study, we used $5\text{-LO}^{-/-}$ mice and pharmacologic tools to evaluate the role of 5-LO products in sepsis-induced lung injury, analyzing lung structure and function as well as local and systemic inflammation.

Materials and Methods

Animals and Protocol

This study was approved by the Ethics Committee of the Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro (IBCCF019), and performed in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

Pathogen-free $5\text{-LO}^{-/-}$ (129-Alox5) and strain-matched WT mice and C57Bl/6 mice were bred in the Laboratory of Transgenic Animals (Federal University of Rio de Janeiro) from breeders from The Jackson Laboratory. Animals (18–20 g) were subjected to CLP surgery as previously described (14). After 16 hours, the animals were anesthetized for evaluation of respiratory mechanics; the animals were killed, and lungs were prepared for histologic analysis and enzymatic activity; and blood and lungs were collected for quantification of mediators.

Pharmacologic Treatments

MK886 (BIOMOL, Plymouth, PA) was orally administered (1 mg/kg) 1 hour before CLP. Montelukast (Cayman Chemicals, Ann Arbor, MI) was administered (1 mg/kg, subcutaneously) 4 hours before and 4 hours after surgery. CP105,696 (a gift from Pfizer, Groton, CT) was administered (3 mg/kg, subcutaneously) 4 hours before and 4 hours after surgery.

Respiratory Mechanics

Animals were sedated with diazepam (1 mg/kg, intraperitoneally), anesthetized with thiopental sodium (20 mg/kg, intraperitoneally), tracheotomized, paralyzed with vecuronium bromide (0.005 mg/kg, intravenously), and ventilated with a constant flow ventilator (Samay VR15; Montevideo, Uruguay) with 100 breaths/min frequency, tidal volume of 0.2 ml, and fraction of inspired oxygen of 0.21. The anterior chest wall was removed, and a positive end-expiratory pressure of 2 cm H_2O was applied. After 10 minutes, lung mechanics were computed. Airflow and tracheal pressure were measured (15). Static lung elastance was computed by the end-inflation occlusion method (16). Mechanics measurements were performed 10 times-per animal. Data were analyzed using ANADAT software (RHT-InfoData, Inc., Montreal, PQ, Canada).

Lung Histology

After lung mechanics, heparin (1,000 IU) was injected intravenously. The trachea was

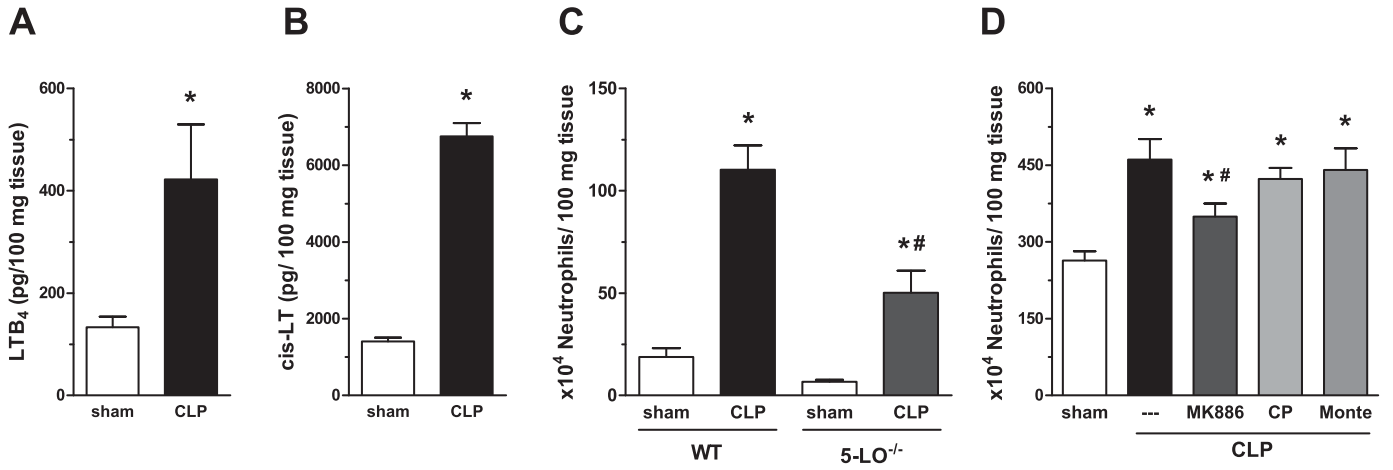

Figure 2. Leukotriene (LT) production and neutrophil influx into the lung after CLP. Concentrations of LTB_4 (*A*) and cys-LTs (*B*) in lung homogenates of C57Bl/6 mice were obtained 16 hours after sham, and CLP was determined by enzyme immunoassay. Neutrophil infiltration in WT and $5\text{-LO}^{-/-}$ mice (*C*) and MK886-, CP 105,696–, and montelukast-treated mice lungs (*D*) was evaluated 16 hours post-sham or CLP by MPO activity. Data are presented as the mean ± SEM. $^{*}P < 0.05$ compared with sham group. $^{\#}P < 0.05$ compared with CLP/WT mice.

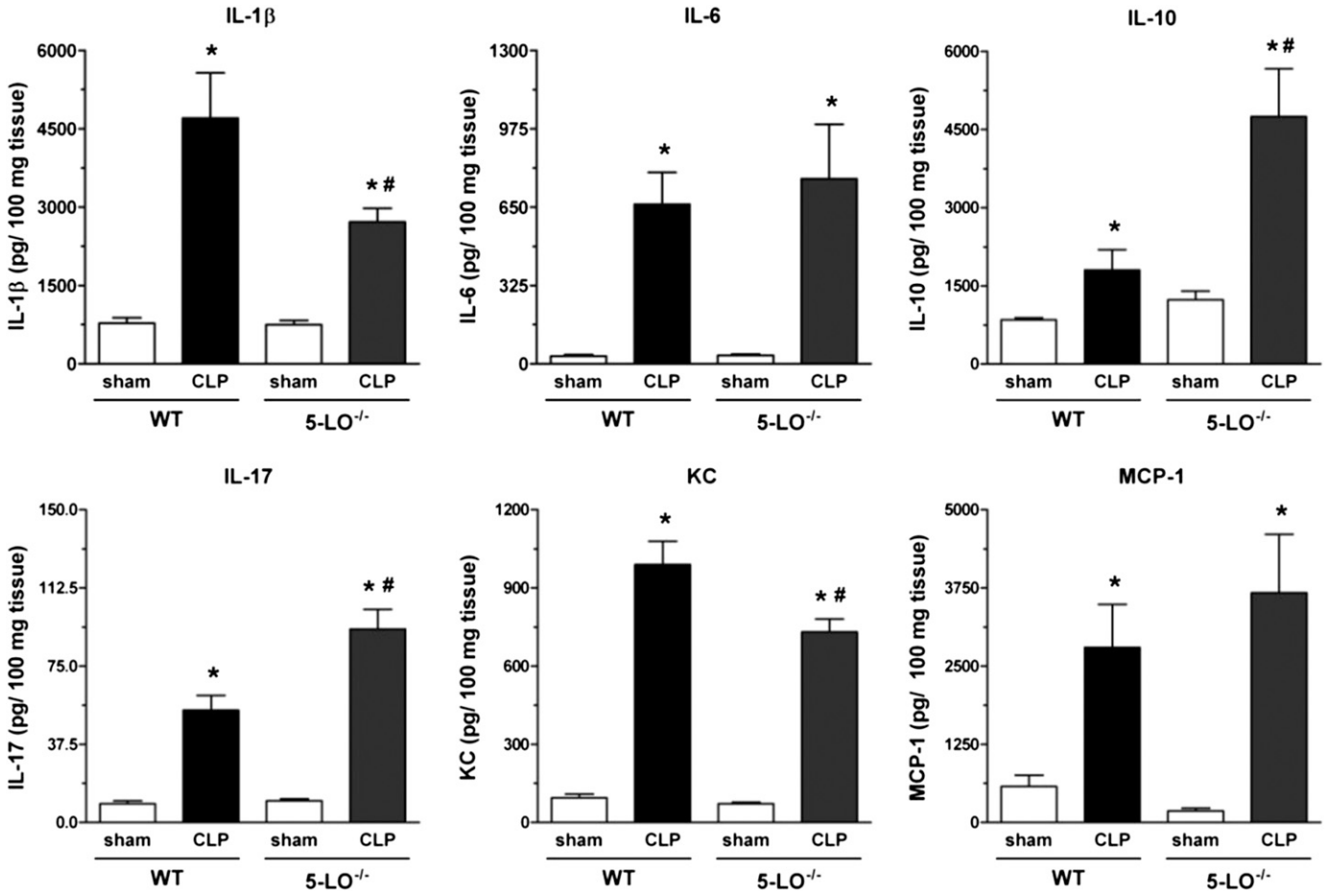

Figure 3. Lung cytokine levels in WT (sv129 strain) and 5-LO$^{-/-}$ mice subjected to sham or CLP. IL-1β, IL-6, IL-10, IL-17, KC, and MCP-1 levels were quantified in lungs 16 hours after sham or CLP of WT and 5-LO$^{-/-}$ mice. Cytokines were measured by ELISA. Data are presented as the mean ± SEM. *$P < 0.05$ compared with sham group. #$P < 0.05$ compared with CLP/WT group.

clamped at end-expiration (positive end-expiratory pressure, 2 cm H_2O), and mice were exsanguinated. The right lung was removed, fixed in 3% buffered formaldehyde, and paraffin embedded. Slices (4 μm thick) were stained with hematoxylin and eosin. Lung morphometry analysis was performed as previously described (17, 18).

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was determined as previously described (19).

Cytokines, LTB_4, cys-LTs, and PGE_2 Analysis

Right lung tissue was placed in 400 μl of cold NaCl/Tris buffer (50 mM/100 mM [pH 8]) with protease inhibitors (Sigma, St Louis, MO), homogenized, and centrifuged (590 *g* for 5 min at 4°C), and supernatants were stored (−80°C) for cytokine and LTB_4 measurement. Plasma was stored (−80°C) for cytokine measurement.

Cytokines were determined by ELISA (BD, Franklin Lakes, NJ), and LTB_4, cys-LTs, and PGE_2 were determined by enzyme immunoassay (Cayman Chemicals) according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was determined by ANOVA with Bonferroni *t* test for unpaired values or Student *t* test as appropriate. Tests were performed using the SPSS version 18.0 (SPSS, Chicago, IL), and significance was set as $P < 0.05$. The results from the experiment are representative of three independent experiments, with six mice per group in each experiment.

Results

Pulmonary Histopathologic Protection in 5-LO$^{-/-}$ Mice after CLP

Lungs from CLP/WT mice exhibited a high degree of inflammatory injury, with cellular accumulation noted predominantly in the interstitium and thickening and edema of the alveolar walls as compared with lungs from sham-operated mice (Figure 1A). CLP/5-LO$^{-/-}$ mice exhibited much less injury and inflammation than CLP/WT animals (Figure 1A). Lung structures of sham-operated WT and 5-LO$^{-/-}$ mice were similar without any evidence of damage. Morphometric analysis demonstrated that sepsis induced collapse of almost 40% of alveoli in WT animals but induced a collapse of only 23.3% in 5-LO$^{-/-}$ mice. The percentage of collapsed alveoli in the lungs of sham-operated groups

was similar in WT and 5-LO$^{-/-}$ mice (Figure 1B).

LT Levels and MPO Activity in the Lungs after CLP

Higher amounts of LTB_4 (Figure 2A), cys-LTs (Figure 2B), and PGE_2 (*see* Figure E1 in the online supplement) were detected in lung tissue of WT animals subjected to CLP compared with sham mice. Having determined that LTs were generated in the lungs during sepsis, we evaluated its contribution to neutrophil accumulation, assessed by MPO activity. WT (sv129) mice subjected to CLP showed higher neutrophil accumulation in the lungs compared with sham-operated animals (Figure 2C). However, CLP-induced neutrophil migration to the lungs was significantly attenuated in 5-LO$^{-/-}$ mice (Figure 2C). This reduction in the numbers of neutrophils was not associated with reduced numbers of neutrophils in peripheral circulation or bone marrow of 5-LO$^{-/-}$ mice because they present similar numbers to those observed in WT (sv129) mice (data not shown). Corroborating this finding, the treatment of C57Bl/6 mice with a FLAP inhibitor (MK886) reduced the number of infiltrating neutrophils in the lungs of animals subjected to CLP (Figure 2C). Receptor antagonism of the high-affinity LTB_4 receptor BLT1 (CP105,696) or the high-affinity cysteinyl receptor cys-LT1 (montelukast), *in vivo* had no such effect (Figure 2D).

CLP-Induced Lung Cytokines: Effect of 5-LO Deletion/Inhibition and Receptor Antagonists

Ample precedent exists for generation of cytokines/chemokines to be influenced by 5-LO pathway products (20–24). Lungs from CLP/WT mice exhibited high quantities of IL-1β, IL-6, IL-10, IL-17, KC, and MCP-1 as compared with sham-operated mice. Lungs from CLP/5-LO$^{-/-}$ mice showed a reduction in levels of IL-1β and KC, no difference in IL-6 and MCP-1, and a significant increase in IL-17 and IL-10 compared with the CLP/WT group (Figure 3). All values observed in sham-operated groups (WT and 5-LO$^{-/-}$) were similar. A similar effect was obtained with MK886, with a decrease in IL-1β and an increase in IL-10. No differences were noted for IL-6, KC, and MCP-1 when compared with untreated mice (Figure 4). The BLT1 and cys-LT1 antagonists had no effect on CLP-induced lung IL-6, KC, and MCP-1. However, both antagonists enhanced IL-1β and IL-10 concentrations after CLP surgery (Figure 4).

CLP-Induced Systemic Cytokines: Effect of 5-LO Deletion/Inhibition and Receptor Antagonists

IL-1β, IL-6, IL-10, IL-17, KC, and MCP-1 levels in plasma from CLP animals were elevated when compared with those obtained from sham-operated animals. As observed in lung tissue, CLP/5-LO$^{-/-}$ mice exhibited a reduction in IL-1β and KC plasma levels, no change in IL-6 and

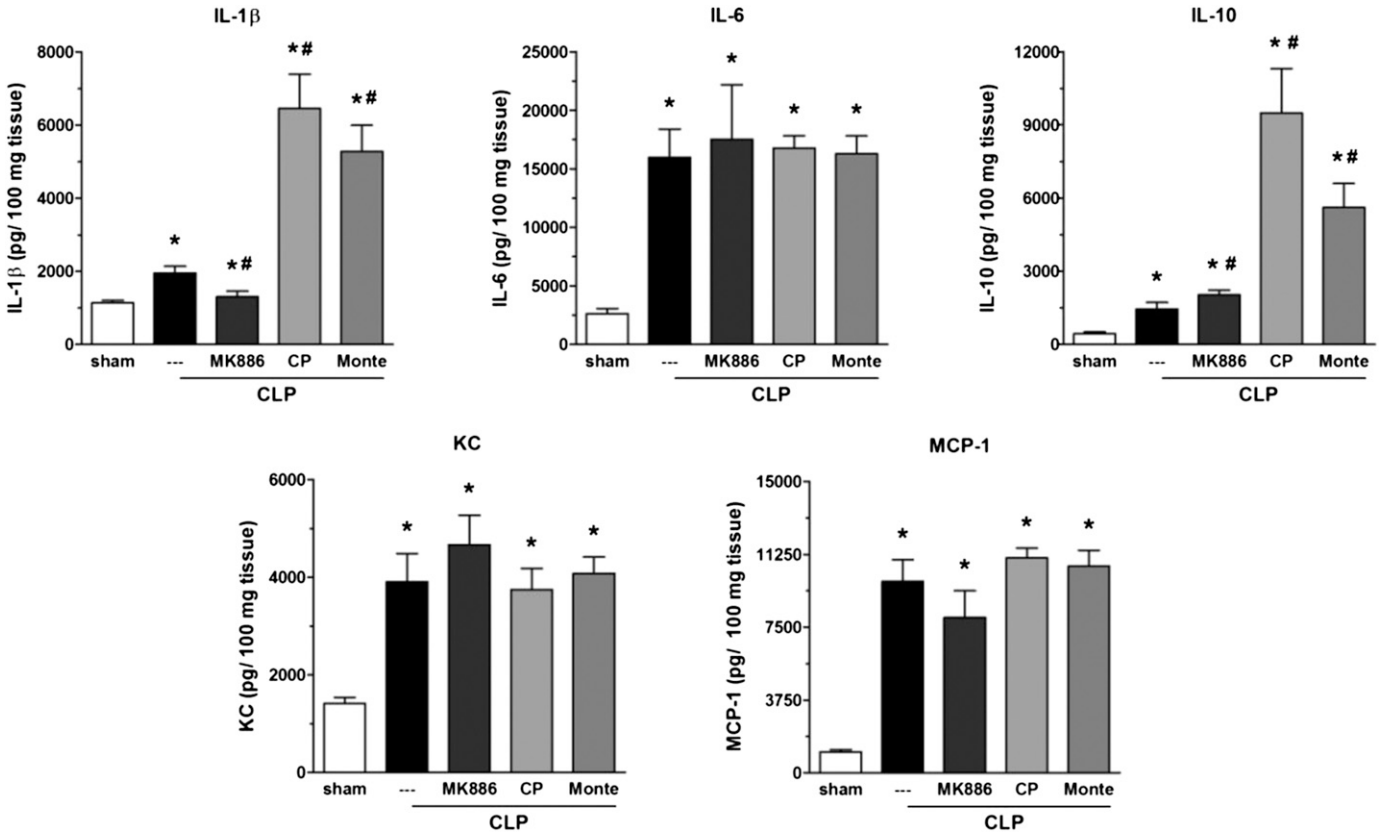

Figure 4. Effect of MK886, BLT1, and cys-LT1 receptor antagonists on lung cytokine levels after CLP. IL-1β, IL-6, IL-10, KC, and MCP-1 levels were quantified in lungs of C57Bl/6 mice 16 hours after sham or CLP in MK886-, CP105,696 (CP)-, or montelukast (Monte)-treated or nontreated mice. Cytokines were measured by ELISA. Data are presented as the mean ± SEM. *$P < 0.05$ compared with sham group. #$P < 0.05$ compared with CLP untreated mice.

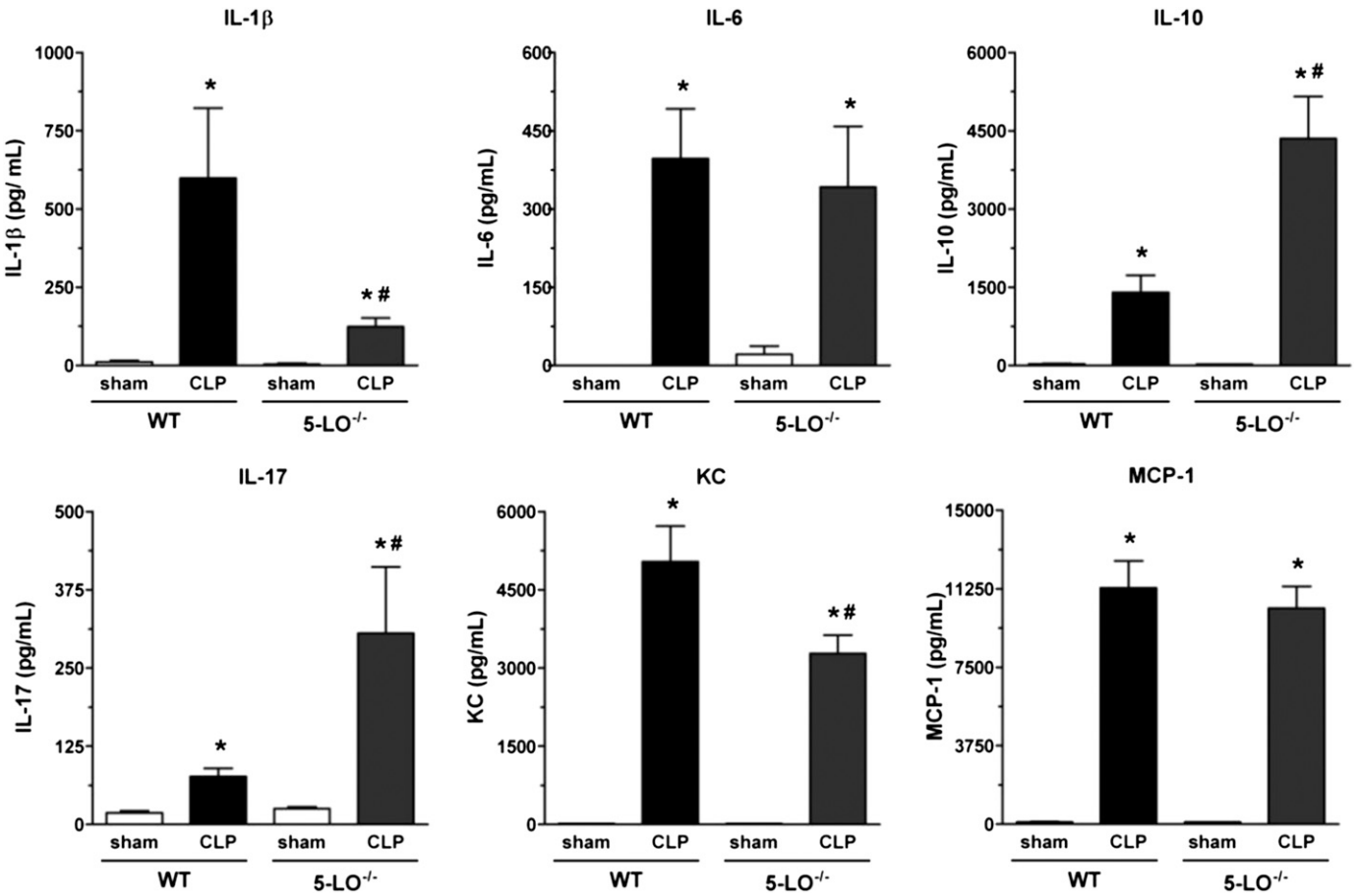

Figure 5. Plasma cytokine levels after sepsis in WT (sv129 strain) and 5-LO$^{-/-}$ mice. IL-1β, IL-6, IL-10, IL-17, KC, and MCP-1 levels were quantified in plasma 16 hours after sham or CLP in WT and 5-LO$^{-/-}$ mice by ELISA. Data are presented as the mean ± SEM. *$P < 0.05$ compared with sham group. #$P < 0.05$ compared with CLP/WT mice.

MCP-1, and increased IL-10 and IL-17 compared with the CLP/WT group (Figure 5). All cytokines analyzed were very low or undetectable in the plasma of sham-operated mice. MK886 treatment also reduced IL-1β and enhanced IL-10 plasma levels compared with the CLP group but had no effect on the other cytokines analyzed (Figure 6). Treatment of CLP mice with CP 105,696 or montelukast evoked an increase in IL-1β and IL-10 when compared with the CLP group without affecting the levels of the other cytokines measured (Figure 6).

Lung Physiologic Protection in 5-LO$^{-/-}$ Mice and in MK886-Treated Animals

CLP/WT mice showed a substantial (63.5%) increase in lung elastance compared with sham-operated animals, consistent with the decrease in compliance during acute respiratory distress syndrome. CLP/5-LO$^{-/-}$ mice exhibited a smaller (21%) increase in lung elastance (Figure 7A). Basal levels of elastance in sham-operated 5-LO$^{-/-}$ mice were modestly higher than those in the control group (WT). The same difference in elastance between WT and 5-LO$^{-/-}$ animals was observed in naive mice (data not shown). Next, we evaluated lung elastance in mice treated with LT synthesis inhibitor (MK886), BLT1 (CP105696), and cys-LT1 (montelukast) receptor antagonists subjected to CLP. CLP-induced lung elastance increase was only inhibited by MK886 treatment and was not altered by CP105696 or montelukast (Figure 7B).

Discussion

Lung injury can occur by direct insults, such as infections, inhalation of toxins, hyperoxia, or aspiration of gastric contents. However, it is also often associated with systemic inflammatory responses, such as burns, trauma, pancreatitis, hemorrhagic shock, and sepsis (25). Lung injury is a severe consequence of sepsis and is one of the most common causes of acute respiratory distress syndrome. Cytokines are well known participants in the inflammatory injury to organs including the lung during sepsis. Although the 5-LO pathway has been implicated in lung injury, including that elicited by LPS administration (26), its role in sepsis-induced lung injury has not previously been examined. Recently, a study demonstrated that a dual inhibitor of cyclooxygenase 2 and 5-LO attenuates the inflammatory response and improves survival in murine CLP, but the independent contributions of these two enzymatic pathways were not distinguished (27). Moreover, no previous studies have determined the role of 5-LO products in the physiologic derangements associated with acute lung injury (ALI).

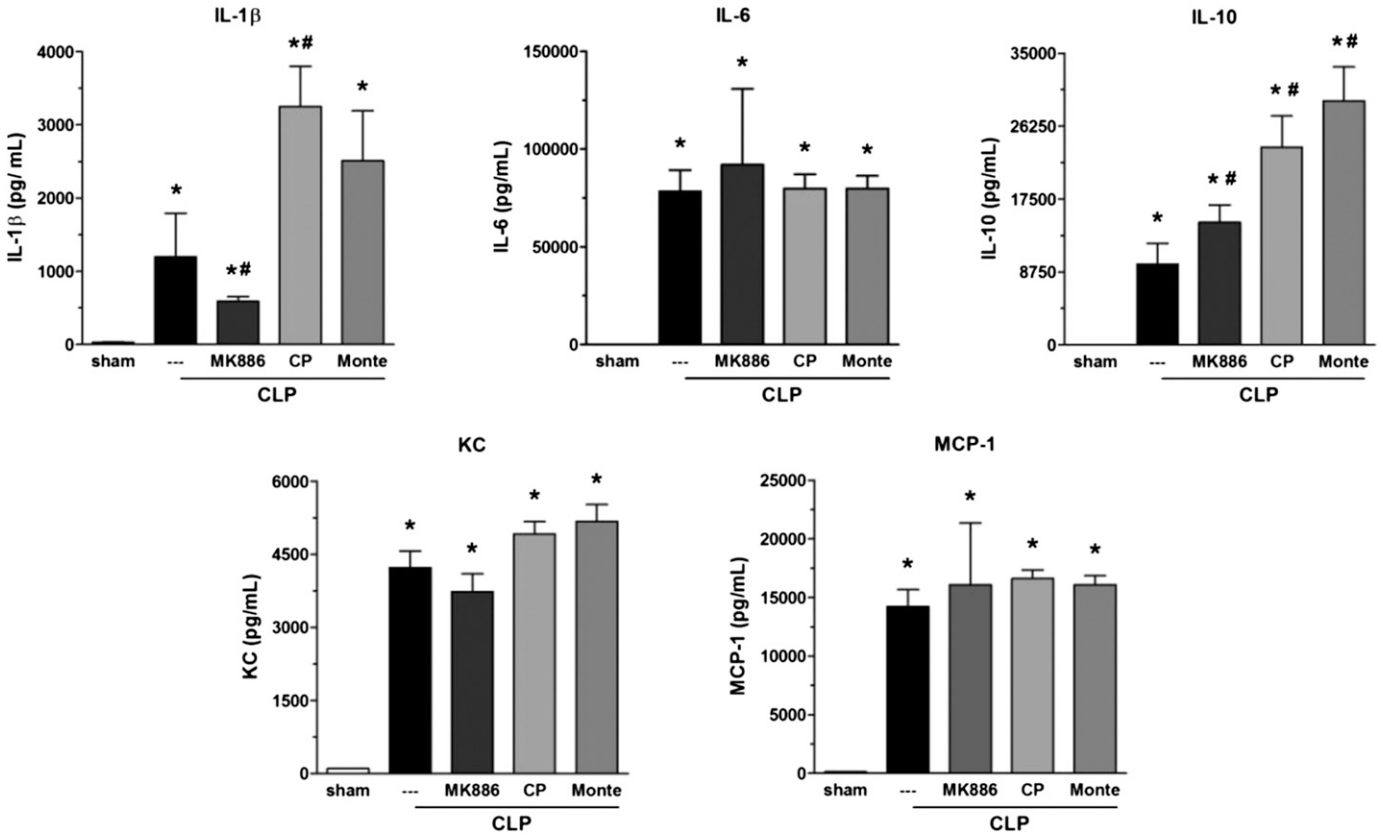

Figure 6. Effects of MK886, BLT1, and cys-LT1 receptor antagonists on plasma cytokine levels after sepsis. IL-1β, IL-6, IL-10, KC, and MCP-1 levels were quantified in lungs 16 hours after sham or CLP in untreated or treated C57Bl/6 mice with MK886, CP105,696 (CP), or montelukast (Monte) by ELISA. Data are presented as the mean ± SEM. $^{*}P < 0.05$ compared with sham group. $^{\#}P < 0.05$ compared with CLP untreated mice.

Our data demonstrate that the morphologic, physiologic, and inflammatory features of the lung injury induced by CLP, a relevant animal model of sepsis, are at least in part attributable to 5-LO products. The results also point to an important role for 5-LO–derived mediators as key modulators of cytokine production in this setting.

CLP-induced lung injury depends on 5-LO–derived mediators. In our study, 5-LO$^{-/-}$ mice showed less morphologic damage and a corresponding protection from increased lung elastance. Further evidence of 5-LO pathway participation was obtained with pretreatment of WT animals with a FLAP inhibitor (MK886), which resulted in less neutrophil recruitment to the lungs and a decrease in IL-1β and KC levels in the lung and plasma and inhibited CLP-induced lung elastance increase. IL-1β and KC are important contributors to neutrophil migration to inflammatory sites (28, 29), and we have previously reported that neutrophil accumulation induced by IL-1β depends on LTB_4 production (24). The importance of IL-1β is emphasized by a recent report highlighting the critical role of inflammasome-regulated cytokines in ventilation-induced ALI in humans (30).

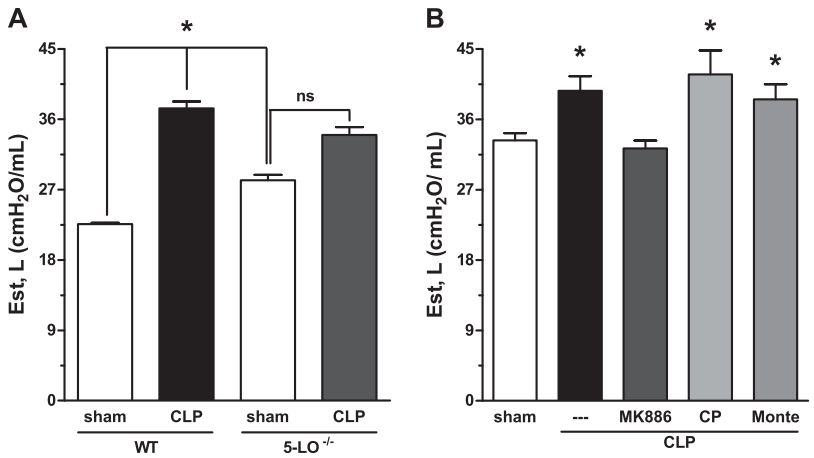

Figure 7. Lung function in 5-LO$^{-/-}$ mice and MK886-treated animals after CLP. Static elastance (Est) was evaluated 16 hours after sham or CLP in WT (sv129 strain) and 5-LO$^{-/-}$ mice (*A*) and in untreated or treated C57Bl/6 mice with MK886, CP105,696 (CP), or montelukast (Monte) (*B*). Data are presented as the mean ± SEM of six animals per group. $^{*}P < 0.05$ compared with sham group. ns = nonsignificant.

Interventions that reduce IL-1β in ALI may be beneficial to the host. It was also recently demonstrated that inflammasome activation can elicit the rapid production of prostaglandins and LTs *in vivo* independently of IL-1β and IL-18 (31). Genetic and pharmacologic disruption of LT biosynthesis led to an increase in IL-10 levels in plasma and in the lungs, and this represents an additional possible protective mechanism because the antiinflammatory actions of IL-10 are well established (32, 33). We also observed a higher production of IL-17 in the lungs and plasma of 5-LO$^{-/-}$ animals subjected to CLP (Figures 4 and 5). There is evidence in the literature that IL-1β can act as an important inducer of Th 17 cells (34, 35), so it was surprising that IL-17 and IL-1β levels tended to be regulated in opposing fashion by 5-LO deletion/inhibition. Although 5-LO$^{-/-}$ mice exhibited higher IL-17 production and IL-17 promotes neutrophil recruitment, we observed a reduction in neutrophil infiltration into lungs in this setting, which may be due to concomitant reductions in IL-1β and KC secondary to reduced LTB_4. IL-17 is involved in autoimmune diseases but has emerged as a critical player in host defense responses and inflammatory diseases (36, 37). It is possible that higher IL-17 levels represent a compensatory effort to better control the infection during polymicrobial sepsis, as previously suggested (38).

Neither genetic nor pharmacologic disruption of LT biosynthesis distinguishes the potential roles of LTB_4, cys-LTs, and other 5-LO metabolites. We therefore treated the animals with selective antagonists for BLT1 and cys-LT1 to evaluate their individual contributions to cytokine modulation. Both of these treatments led to an increase in IL-1β and IL-10 production. These results contrast with those obtained by targeting the biosynthetic 5-LO–FLAP step and point to complex actions and interplay between BLT1 and cys-LT1 receptors. Furthermore, the possible participation of other 5-LO products (e.g., 5-HETE), not evaluated in this study, is not ruled out. Although our results suggest that 5-LO products participate in the pathogenesis of sepsis-induced lung injury, specific LT receptor blockade was substantially less protective than 5-LO inhibition. Several possible explanations exist for this discrepancy. First, the 5-LO and cyclooxygenase enzymes can compete for their common substrate, arachidonic acid. Shunting of arachidonic acid through the cyclooxygenase pathway leading to an increased production of PGE_2 has been observed in macrophages from 5-LO$^{-/-}$ mice (39, 40), and increased lung lavage PGE_2 levels have been reported in 5-LO$^{-/-}$ mice (41). PGE_2 is able to inhibit leukocyte recruitment (42) and the production of IL-8 (43) and TNF-α (44) and to promote IL-10 synthesis (45). Another possible explanation relates to the fact that the antagonists used specifically antagonize the high-affinity receptors BLT1 and cys-LT1 but not the low-affinity receptors BLT2 and cys-LT2. These findings are in accordance with our previous report (8), in which resistance against CLP-induced mortality was observed in 5-LO$^{-/-}$ mice or WT animals treated with the FLAP inhibitor (MK886) but not in animals treated with a BLT1 antagonist. In that study, a cys-LT1 antagonist conferred a moderate degree of protection against lethality, which was attributed to less sepsis-induced hypotension. Furthermore, the data presented here and elsewhere (8) support that, independent of the mouse strain used (sv129 or C57Bl/6), we were able to observe relevant protective effects during 5-LO inhibition or deletion but not with the use of receptor antagonists alone.

The increased baseline elastance in 5-LO$^{-/-}$ mice may represent an intrinsic characteristic of 5-LO$^{-/-}$ lungs. Differences in quantities and/or types of matrix proteins may exist, but this requires further characterization. Nevertheless, 5-LO$^{-/-}$ mice and MK886-treated mice were clearly protected from further elevation in elastance resulting from CLP.

In conclusion, our data suggest that 5-LO products contribute to sepsis-induced lung injury, and we speculate that 5-LO inhibition could be beneficial as a strategy to dampen inflammatory responses observed after sepsis. However, 5-LO products promote innate immune responses, and blocking this pathway could interfere with host defense. For this reason, the use and timing of 5-LO inhibitors to impair sepsis-induced lung injury must be carefully considered. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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