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# Opposing and Hierarchical Roles of Leukotrienes in Local Innate Immune versus Vascular Responses in a Model of Sepsis<sup>1</sup>

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The 5-lipoxygenase (5-LO)-derived leukotrienes (LTs) influence both local innate immunity and vascular responses, but the relative importance of effects on these two processes in sepsis is unknown. In a cecal ligation and puncture model of peritonitis with severe sepsis, 5-LO<sup>-/-</sup> mice showed a reduction in peritoneal neutrophil accumulation and an increase in the number of bacteria in the peritoneal cavity. Despite this impairment of local innate immunity, the null mice exhibited a marked improvement in survival, and this protection was also seen in wild-type animals treated with the LT synthesis inhibitor MK 886. A survival advantage in severe sepsis was also observed in mice treated with the cysteinyl-LT receptor antagonist MK 571, but not with the LTB<sub>4</sub> receptor antagonist CP 105, 696. Protection in the 5-LO<sup>-/-</sup> mice was associated with reduced vascular leak and serum lactate levels. Moreover, wild-type mice treated with MK 571 exhibited less sepsis-induced hypotension. These data demonstrate opposing effects of cysteinyl-LTs on innate immune vs hemodynamic responses, demonstrating protective effects on local immunity and deleterious effects on the vasculature. They also suggest the possible therapeutic utility of targeting vascular events in sepsis with cysteinyl-LT blockade. *The Journal of Immunology*, 2005, 174: 1616–1620.

When local innate immunity is overwhelmed and microbial infection disseminates via the bloodstream, the sepsis syndrome, characterized by hypotension, poor tissue perfusion, and multiorgan dysfunction, results. Because of increases in interventional procedures, immunosuppression, and antibiotic resistance, the incidence of sepsis has risen rapidly, with >500,000 cases annually in the U.S. alone. Despite the availability of an increasing array of potent antibiotics and intensive medical care, mortality in sepsis remains high (1–3). A myriad of investigational therapies has had little impact on outcomes in sepsis, and novel approaches are required.

Leukotrienes (LTs)<sup>4</sup> are proinflammatory mediators best known for their role in the pathogenesis of asthma, but recently implicated in a variety of other disease states, including pulmonary fibrosis, arthritis, and atherosclerosis (4, 5). LTs are derived from the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism. The enzyme 5-LO, in conjunction with its helper protein 5-LO-activat-

ing protein (FLAP), oxygenates arachidonic acid to form LTA<sub>4</sub>. This unstable intermediate can be hydrolyzed to form the potent leukocyte activator and chemoattractant LTB<sub>4</sub>, or conjugated with glutathione to form the spasmogenic cysteinyl-LTs (cys-LTs; LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>), comprising the activity previously known as slow reacting substance (6).

An important role for endogenous LTs in antimicrobial defense was suggested by our previous report that 5-LO<sup>-/-</sup> mice exhibited enhanced lethality and reduced pulmonary bacterial clearance, as compared with their wild-type (WT) counterparts, in a model of *Klebsiella pneumoniae* pneumonia (7). Subsequent studies demonstrated that both classes of LTs were necessary for optimal phagocytosis of IgG-opsonized microbes (7–9).

In addition to their actions on airways, cys-LTs have long been recognized to increase microvascular permeability in various organs (6, 10, 11). Exclusively on the basis of studies using intravascular bolus injection of either live bacteria or bacterial endotoxin, cys-LTs have been suggested to participate in the vascular manifestations of septic shock (12, 13). However, we know of no studies that have explored this possibility using a more clinically relevant model of septic shock in which local innate immunity is challenged. Because it is only recently that roles of LTs in innate immunity have been recognized, evaluating the potential utility of cys-LT antagonism in septic shock requires a consideration of the roles of these lipids in both local innate immunity as well as vascular responses to disseminated infection.

In the present study, we sought to determine the role of LTs in innate immunity and vascular events in a relevant experimental model of peritonitis and polymicrobial sepsis. Collectively, our findings demonstrate that cys-LTs have divergent effects during the evolution of sepsis. Initially, as is true for LTB<sub>4</sub>, they participate in local innate immune control. However, if the severity of infection overwhelms local immunity and microbial dissemination ensues, cys-LTs (but not LTB<sub>4</sub>) contribute to the deleterious

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<sup>4</sup> Abbreviations used in this paper: LT, leukotriene; CLP, cecal ligation and puncture; Cys-LT, cysteinyl-LT; FLAP, 5-lipoxygenase-activating protein; 5-LO, 5-lipoxygenase; WT, wild type.

effects on the vasculature, resulting in vascular leak, hypotension, and inadequate tissue perfusion.

## Materials and Methods

### Mouse model of cecal ligation and puncture (CLP)

Specific pathogen-free female or male 5-LO<sup>-/-</sup> (129-Alox5<sup>tm1Fur</sup>) (14) and strain-matched WT mice were bred in the University of Michigan Unit for Laboratory Animal Medicine from breeders obtained from The Jackson Laboratory. The animals (18–20 g) were subjected to CLP surgery, as previously described in detail (15). Briefly, mice were anesthetized by the i.p. injection of a mixture of ketamine (ketamine hydrochloride, 112.5 mg kg<sup>-1</sup>; Abbott Laboratories) and xylazine (Anased, 7.5 mg kg<sup>-1</sup>; Lloyd Laboratories). Under sterile surgical conditions, a 1- to 2-cm midline incision was made on the ventral surface of the abdomen, and the cecum was fully exposed through this incision. The cecum was ligated (without causing bowel obstruction) at its base with a 4-0 silk suture, and punctured a total of eight times with a 21-gauge needle. Sham-operated mice underwent an identical laparotomy, but did not undergo ligation and punctures and served as controls. In both groups of mice, the abdominal incision was closed using a surgical staple, and 1 ml of sterile normal saline was administered s.c. for fluid resuscitation just after surgery. Survival was monitored in groups of mice subjected to a sham operation or CLP surgery for up to 7 days after these procedures. The numbers of mice used in each experiment are noted in the figures.

### Neutrophil migration into the peritoneal cavity after CLP surgery

Neutrophil migration was evaluated at 6 h after sham or CLP surgery. Mice were euthanized by an anesthesia overdose, and the cells present in the peritoneal cavity were harvested by lavage with 3 ml of sterile PBS buffer. Total counts were determined using a hemocytometer, and differential cell counts (200 cells total) were conducted on cytocentrifuged (Cytospin; Thermo Shandon) slides stained with Diff-Quick. The results are presented as the number of neutrophils per peritoneal cavity.

### Bacterial counts in the peritoneal cavity

At 48 h after sham operation or CLP, animals were sacrificed by an anesthesia overdose and the peritoneal cavity was lavaged. Aliquots of serial log dilutions of the peritoneal lavage fluid were plated on tryptic soy agar dishes (Difco Laboratories). CFU were counted after overnight incubation at 37°C, and the results were expressed as CFU per cavity.

### Protein extravasation

Protein extravasation was assessed, as previously described (16, 17). Briefly, 5 h after sham or CLP surgery, mice were injected i.v. with Evans blue dye (Sigma-Aldrich; 1 mg per mouse in a volume of 0.2 ml). One hour later, anesthetized mice were perfused with heparinized saline via the left ventricle, and the peritoneal exudates, kidneys, and hearts were recovered. The peritoneal exudates were centrifuged for 10 min at 200 × g, and the supernatant was saved for colorimetric determinations. Each tissue sample was dried, weighed, and placed in 1 ml of formamide at 37°C for 24 h. The formamide samples were then centrifuged, and the OD of the supernatant was determined at 630 nm. The values obtained were normalized by gram of tissue, and expressed as μg per cavity or per gram of tissue based on a standard curve.

### Lactic acid analysis

The plasma was collected in Na fluoride/potassium oxalate tubes (BD Biosciences vacutainer), and the samples were analyzed following manufacturer description by Vitros Test Methodologies (J&J Clinical Diagnostics; LAC Test Methodology) in the Chemistry section, Department of Pathology, University of Michigan Hospital. Lactic acid analysis was based on the conversion of lactate to pyruvate and hydrogen peroxide in the presence of oxygen and the catalyst lactate oxidase. The resulting hydrogen peroxide reacts with a chromogen to produce a red dye. Its intensity is determined at 540 nm by reflectance spectrophotometry, and is related to the concentration of lactate in the specimen. The results were expressed as mmol L<sup>-1</sup>.

### Noninvasive systolic blood pressure determination

The systolic blood pressure was measured using a modification (18) of the tail-cuff procedure originally described by Krege et al. (19) and manufacturer's instruction manual. Briefly, mice were acclimated for 3 days to a restraint platform (33–34°C) and to tail-cuff inflation. A traditional tail-cuff occluder was placed on the mouse's tail between a light source above and

a photoresistor below. Upon inflation, the occluder stopped blood flow through the tail, and upon deflation, the return of blood flow was detected by the sensor. An initial series of inflation-deflation cycles was used to calibrate the amplifier (Visitech, Model BP 2000 Blood Pressure Analysis System; Visitech Systems), which automatically takes ten 30-s measurements using proprietary software (BP-2000 Software β Version 03/10/97). If at least 8 of the 10 readings were acceptable, the highest and lowest readings were discarded and the remaining readings were averaged to yield a single value.

### PGE<sub>2</sub> determination

Cell-free peritoneal exudates were collected following sham or CLP surgery and were analyzed by enzyme immunoassay for the predominant cyclooxygenase product PGE<sub>2</sub>, using a commercially available kit from Assay Designs.

### Administration of pharmacological agents

In some experiments, the mice were pretreated with inhibitors of LT synthesis or actions. MK 886 (BIOMOL), a FLAP inhibitor, was orally administered by gavage (1 mg kg<sup>-1</sup>) twice daily for 2 days, with a final dose 10 min before surgery. MK 571 (BIOMOL), a cys-LT receptor 1 antagonist, was orally administered at a dose of 20 mg kg<sup>-1</sup> 1 h before surgery and twice daily for the subsequent 3 days. CP 105, 696, a LTB<sub>4</sub> receptor 1 antagonist, was a generous gift from H. Showell (Pfizer, Groton, CT). CP 105, 696 was injected s.c. at a dose of 3 mg kg<sup>-1</sup> 30 min before surgery and twice daily for the subsequent 3 days. These doses were selected from previous work and literature (20, 21).

### Statistical analysis

Data are presented as means ± SE and are representative of two to four separate experiments. The mean values of different treatments were compared by ANOVA. If significance was detected, individual differences were evaluated using Bonferroni's *t* test for unpaired values. Statistical significance was set at *p* < 0.05. Survival rates were expressed as percentages, and a log rank test ( $\chi^2$  test) was used to detect differences in mouse survival. The statistical analysis of the frequency of bacterial peritonitis was evaluated using  $\chi^2$  test.

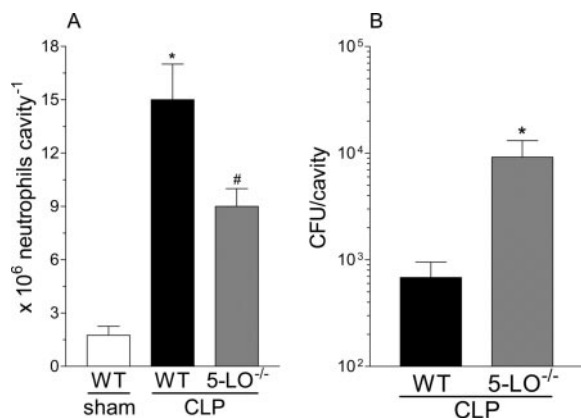
## Results

### The 5-LO<sup>-/-</sup> mice exhibit a diminished innate immune response to bacterial peritonitis

Neutrophil counts and bacterial cultures of the peritoneal exudates were evaluated in WT and 5-LO<sup>-/-</sup> mice 6 and 48 h, respectively, after surgery. The 5-LO<sup>-/-</sup> mice subjected to CLP exhibited significantly less peritoneal neutrophil accumulation than did WT mice (Fig. 1A). This was accompanied in the 5-LO<sup>-/-</sup> mice by a significant increase in the percentage of mice with positive bacterial cultures in the peritoneal lavage fluid (data not shown) as well as by an increase in the number of CFUs (Fig. 1B), as compared with WT mice.

### Genetic and pharmacologic inhibition of 5-LO confers resistance against CLP-induced mortality

Considering the role of LTB<sub>4</sub> as a neutrophil chemoattractant and the importance of LTs for optimal leukocyte activation and phagocytosis of pathogens (7–9, 13), the findings (Fig. 1) that 5-LO<sup>-/-</sup> mice manifest impaired local neutrophil migration and bacterial clearance were not surprising. However, it was surprising that despite such impairment of local immune responses, the 5-LO<sup>-/-</sup> mice were resistant to the lethal effects of a severe form of CLP characterized by ~75% mortality (Fig. 2). Furthermore, WT mice treated with the inhibitor of FLAP and of LT biosynthesis, MK 886, were likewise protected against the lethality of CLP (Fig. 2). Of note, all animals subjected to sham operation survived (data not shown). As an internal control, we also treated 5-LO<sup>-/-</sup> mice with MK 886, and these animals also demonstrated 100% survival after CLP (data not shown). These data suggest that products of 5-LO mediated not only local immunity, but also systemic responses, contributing to lethality observed in sepsis.



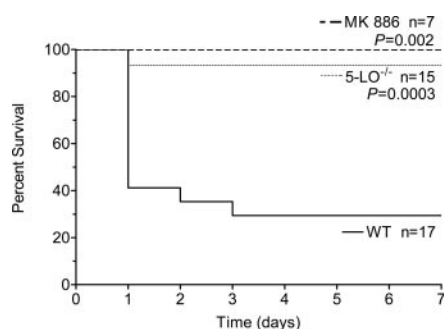
**FIGURE 1.** Neutrophil counts and bacterial cultures of the peritoneal exudates from WT and 5-LO<sup>-/-</sup> mice after CLP surgery. *A*, WT and 5-LO<sup>-/-</sup> mice were subjected to sham or CLP surgery, and neutrophil recruitment to the peritoneal cavity was evaluated 6 h later. The results are expressed as mean  $\pm$  SEM,  $n = 5-8$  animals per group. The data are from one experiment that is representative of a second. \*,  $p < 0.05$  compared with WT sham-operated group. #,  $p < 0.05$  compared with WT mice subjected to CLP. *B*, WT ( $n = 9$ ) and 5-LO<sup>-/-</sup> ( $n = 10$ ) mice were subjected to CLP surgery, and the peritoneal exudates were evaluated for CFU 48 h thereafter. The results are expressed as CFU per cavity, and the data are from one experiment that is representative of three separate experiments.

#### Inhibition of protein extravasation in 5-LO<sup>-/-</sup> mice

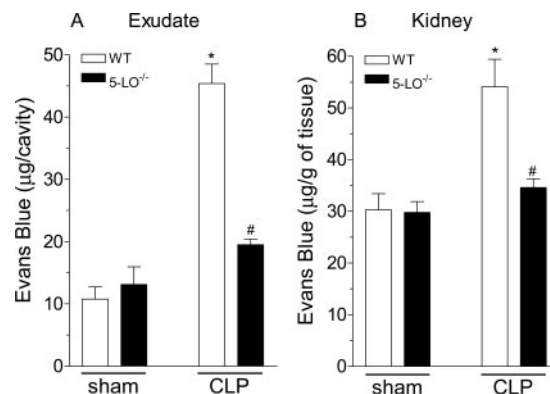
Because LTs are known to have vascular effects, we compared the vascular permeability of WT and 5-LO<sup>-/-</sup> mice subjected to sham operation or severe CLP. WT mice subjected to CLP manifested a marked increase in protein leak into the peritoneal exudates compared with WT mice subjected to sham operation. The 5-LO<sup>-/-</sup> mice showed significantly reduced local peritoneal vascular permeability after CLP surgery (Fig. 3A). Similarly, 5-LO<sup>-/-</sup> mice also demonstrated less protein extravasation in the kidney (Fig. 3B) and heart (WT,  $21 \pm 0.9 \mu\text{g/g}$ ,  $n = 4$ ; 5-LO<sup>-/-</sup>,  $11.9 \pm 0.9 \mu\text{g/g}$ ,  $n = 5$ ) when compared with WT animals. It is important to note that the degree of protein leak observed in 5-LO<sup>-/-</sup> mice subjected to CLP was similar to that noted in sham-operated mice, reflecting a near-complete protection against protein extravasation.

#### The 5-LO<sup>-/-</sup> mice are protected from CLP-induced lactic acidosis

Vascular leak and hypotension in sepsis result in impaired organ perfusion and subsequent accumulation of lactic acid in the serum.



**FIGURE 2.** Survival of LT-sufficient (WT) and LT-deficient (5-LO<sup>-/-</sup>) mice in severe sepsis. WT or 5-LO<sup>-/-</sup> mice were treated with or without MK 886, as described in *Materials and Methods*. Animal survival was followed for 7 days after CLP, and the results were expressed as survival percentage. The number of animals in each group is shown in the figure. Values of  $p$  represent the comparison with untreated WT mice.

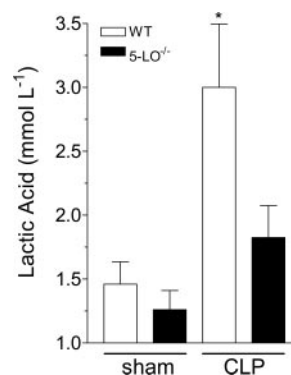


**FIGURE 3.** Protein extravasation in the peritoneal exudates and kidney of WT and 5-LO<sup>-/-</sup> mice subjected to sham or CLP surgery. Animals were injected with Evans blue and then sacrificed 5 and 6 h, respectively, after surgery. Peritoneal exudates (*A*) and kidney (*B*) were collected and prepared, as described in *Materials and Methods*, for the colorimetric assay of extravascular dye leak. The results were normalized for  $\mu\text{g cavity}^{-1}$  and  $\mu\text{g g}^{-1}$  of tissue for the exudates and kidney, respectively. The results are expressed as mean  $\pm$  SEM,  $n = 5-6$  animals per group. Data are from one experiment that is representative of three separate experiments. \*,  $p < 0.05$  compared with WT sham-operated group; #,  $p < 0.05$  compared with WT CLP-operated mice.

Serum lactic acid levels are known to correlate with mortality after sepsis (22). We therefore measured serum lactate levels 24 h after sham or CLP surgery. WT animals demonstrated a significant increase in lactate levels after CLP surgery (Fig. 4). However, 5-LO<sup>-/-</sup> mice subjected to CLP exhibited lactate levels that were lower than WT mice, and similar to those obtained in sham-operated mice. This finding reinforces the role of 5-LO products in the vascular responses to CLP.

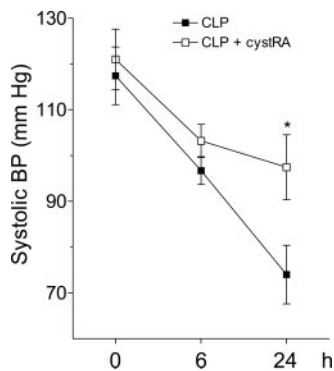
#### WT mice treated with a cys-LT receptor antagonist exhibited less sepsis-induced hypotension

Neither genetic nor pharmacologic disruption of LT biosynthesis distinguishes the role of cys-LTs from other 5-LO metabolites. Among the 5-LO metabolites, cys-LTs are the most likely to exert direct effects on vascular tone and permeability, and therefore to participate in the hemodynamic compromise of sepsis (23, 24). To evaluate their distinct role, WT mice were treated with the cys-LT receptor antagonist MK 571 before CLP surgery, and systolic



**FIGURE 4.** Lactic acid levels in WT and 5-LO<sup>-/-</sup> mice subjected to CLP surgery. Blood was collected 24 h after surgery, and plasma lactic acid content was analyzed, as described in *Materials and Methods*. The results are expressed as mean  $\text{mmol L}^{-1} \pm$  SEM,  $n = 5-6$  animals per group. Data are from one experiment that is representative of three separate experiments. \*,  $p < 0.05$  compared with WT sham-operated mice.





**FIGURE 5.** Effect of cys-LT receptor antagonist on sepsis-induced hypotension. Systolic blood pressure of untreated and MK 571-treated WT mice was evaluated noninvasively, as described in *Materials and Methods*, before and 6 and 24 h after CLP surgery. The results are expressed as mean  $\pm$  SEM,  $n = 4$  animals per group. Data are from one experiment that is representative of two separate experiments. \*,  $p < 0.05$  compared with untreated mice.

blood pressure was evaluated noninvasively. As shown in Fig. 5, untreated WT mice exhibited a progressive decline in systolic blood pressure over 24 h following CLP. Mice treated with MK 571 exhibited significantly less sepsis-induced hypotension. These data support the idea that cys-LTs are important determinants of the hemodynamic response to sepsis.

#### Effect of $LTB_4$ and cys-LT receptor antagonists on survival

The effects of specific LT receptor antagonists on survival were examined in severe lethal sepsis (Fig. 6). Under conditions in which control mice exhibited 90–100% lethality, a moderate, but statistically significant, protection was observed after treatment with the cys-LT receptor antagonist MK 571 (Fig. 6A); however, no protection was afforded by the  $LTB_4$  receptor antagonist CP 105, 696 (Fig. 6B).

## Discussion

In the present study, we extended our exploration of the mechanisms by which LTs modulate the innate immune and vascular responses during sepsis. Sepsis is a complex response, and among the many local and systemic mediators contributing to its expression, roles for cys-LTs as well as  $LTB_4$  have already been demonstrated (4, 25, 26). However, this is the first study to specifically address the roles of cys-LTs and  $LTB_4$  in innate immune vs hemodynamic responses in a relevant experimental model of sepsis. Our data provide the novel insight that cys-LTs play pivotal, yet

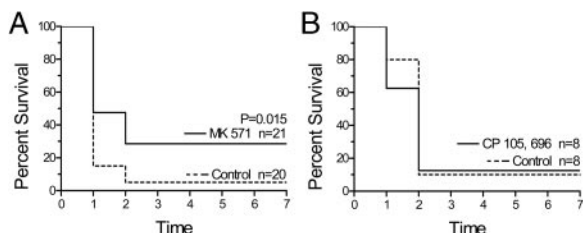
opposing roles in local immune defense and in systemic vascular responses.

Wild-type mice subjected to CLP have been reported to contain high levels of  $LTB_4$  in the peritoneal lavage fluid (27). For this reason, we sought to manipulate the 5-LO pathway, by both genetic and pharmacologic approaches, to investigate the roles of its metabolites in the development of sepsis. The 5-LO inhibition or deletion reduced peritoneal neutrophil accumulation and increased the number of bacteria in the peritoneal cavity, but surprisingly, protected against the lethal effects of CLP.

Protein extravasation revealed that 5-LO null mice were locally (peritoneal cavity) and systemically (kidney and heart) protected from CLP-induced protein leak to a marked degree (Fig. 3). Furthermore, 5-LO<sup>-/-</sup> mice subjected to CLP manifested serum lactate levels that were lower than WT mice (Fig. 4). One key component of sepsis is the manifestation of hypotension, leading to multiple organ failure syndrome and death (18, 28, 29). Hypotension is closely related to the loss of fluid from the vasculature with increased vascular permeability. Because cys-LTs are the most likely 5-LO products to exert direct effects on vascular tone and permeability, their absence was likely to account for the protective effect observed with 5-LO depletion. Indeed, we demonstrated that mice treated with a specific antagonist for cys-LT receptor 1, MK 571, exhibited significantly less sepsis-induced hypotension (Fig. 5). Taken together, our data suggest that cys-LTs are important mediators of the hypotension observed after CLP surgery, and this effect probably reflects enhanced vascular permeability.

To determine whether the advantage in survival observed in 5-LO<sup>-/-</sup> mice and in mice treated with a LT synthesis inhibitor (MK 886) was in fact specifically due to the inhibition of cys-LT synthesis, we investigated the effects of specific receptor antagonists for  $LTB_4$  and cys-LTs on the survival of mice subjected to CLP. In severe lethal sepsis, no survival advantage was afforded by the  $LTB_4$  receptor antagonist CP 105, 696; however, a moderate protection was observed after treatment with the cys-LT receptor antagonist MK 571.

Although our results clearly suggest that cys-LTs are the 5-LO products that account for the vasculopathic effects in CLP sepsis, specific cys-LT receptor 1 blockade was substantially less protective than was complete 5-LO inhibition (compare Figs. 2 and 6). Several possible explanations can be offered for this discrepancy. The 5-LO and cyclooxygenase enzymes can compete for the metabolism of their common substrate, arachidonic acid. Shunting of arachidonic acid through the cyclooxygenase pathway with increased production of  $PGE_2$  has been variably observed in cultured peritoneal macrophages from 5-LO knockout mice (14, 30), and increased lung lavage  $PGE_2$  levels have been reported in 5-LO knockout mice (31). As  $PGE_2$  is itself capable of inhibiting leukocyte recruitment (32), inhibiting production of proinflammatory cytokines including IL-8 (33) and TNF- $\alpha$  (34), and promoting the synthesis of IL-10 (35), this was a relevant consideration. However, we ruled out this possibility by demonstrating no difference between WT and 5-LO<sup>-/-</sup> animals in the measured levels of  $PGE_2$  in peritoneal exudates obtained from sham- and CLP-operated mice (data not shown). Another possible explanation relates to the fact that MK 571 is a specific antagonist of cys-LT receptor 1, but not of cys-LT receptor 2. As evidence has implicated cys-LT receptor 2 in certain vascular events (36), it is possible that some of the vascular effects of cys-LTs in sepsis likewise are mediated via cys-LT receptor 2; further studies are required to define a possible role of cys-LT receptor 2 in sepsis. Finally, although  $LTB_4$  does not exert direct effects on vascular responses, we cannot rule out the possibility of indirect vascular effects of this mediator.  $LTB_4$  induces IL-6, superoxide anions (37), thromboxane, and histamine



**FIGURE 6.** Effect of  $LTB_4$  and cys-LT receptor antagonists on survival in severe sepsis. *A*, Untreated WT mice or mice treated with cys-LT receptor antagonist MK 571 were subjected to CLP, and survival followed for 7 days. *B*, Untreated WT mice or mice treated with  $LTB_4$  receptor antagonist CP 105, 696 were subjected to CLP, and survival was evaluated for 7 days. Statistically significant differences from control are indicated in the figure.

(38), all of which might be vasoactive. Moreover, it was demonstrated that LTA<sub>4</sub> hydrolase-deficient mice, which are unable to synthesize LTB<sub>4</sub>, but can synthesize cys-LTs, exhibited diminished ear edema and extravasation of plasma proteins after topical application of arachidonic acid (39). One or more of these mechanisms might permit LTB<sub>4</sub> to amplify the actions of cys-LTs on the vasculature in sepsis.

Given the critical need to identify new modes of therapy for sepsis, it is attractive to consider the therapeutic potential of cys-LT antagonists. These agents could be rapidly implemented because of the current availability of agents approved for the treatment of asthma. Our data provide support for future investigations of the role of LTs in sepsis pathogenesis and possible therapeutic targeting of this pathway.

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