Treatment with BX471, a CC chemokine receptor 1 antagonist, attenuates systemic inflammatory response during sepsis

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1Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; 2Department of Immunology, Berlex Biosciences, Richmond, California; and 3Defense Science Organization National Laboratories, Singapore
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He M, Horuk R, Moochhala SM, Bhatia M. Treatment with BX471, a CC chemokine receptor 1 antagonist, attenuates systemic inflammatory response during sepsis. Am J Physiol Gastrointest Liver Physiol 292: G1173–G1180, 2007. First published January 18, 2007; doi:10.1152/ajpgi.00420.2006.—Sepsis is a complex clinical syndrome resulting from a harmful host inflammatory response to infection. Chemokines and their receptors play a key role in the pathogenesis of sepsis. BX471 is a potent nonpeptide CC chemokine receptor-1 (CCR1) antagonist in both human and mouse. The aim of the present study was to evaluate the effect of prophylactic and therapeutic treatment with BX471 on cecal ligation and puncture-induced sepsis in the mouse and to investigate the underlying mechanisms. In sepsis induced by cecal ligation and puncture, treatment with BX471 significantly protected mice against lung and liver injury by attenuating MPO activity, an indicator of neutrophil recruitment in lungs and livers and attenuating lung and liver morphological changes in histological sections. Blocking CCR1 by BX471 also downregulated ICAM-1, P-selectin, and E-selectin expression at mRNA and protein levels in lungs and livers compared with placebo-treated groups. These findings suggest that blockade of CCR1 by specific antagonist may represent a promising strategy to prevent disease progression in sepsis.

acute respiratory distress syndrome; multiple organ dysfunction syndrome; systemic inflammatory response syndrome

Sepsis is a complex clinical syndrome resulting from a harmful host response to infection, which frequently occurs after hemorrhage, trauma, burn, or abdominal surgery (4). Epidemiological study shows that in the United States more than 500,000 patients develop sepsis per year. It has been estimated that the annual costs for treatment of sepsis are $16.7 billion in USA alone (20). The mortality rate is approximately between 30 and 70% (19).

The host response to pathogens in sepsis is characterized by infiltration of specific leukocyte populations into host tissues. It is known that this process is predominantly mediated by a family of cytokines: chemokines (10). The chemokines are small 8- to 10-kDa proteins. Over 40 chemokines have been identified to date. They can be subdivided into four families on the basis of the relative position of cysteine residues. Two major subfamilies, CXC chemokines and CC chemokines, have been extensively investigated in inflammatory diseases (28). Chemokines bind to a family of seven-transmembrane domain G protein-coupled receptors on the surface of leukocytes. Nearly 20 different types of chemokine receptors have been described. CC chemokine receptor-1 (CCR1) is expressed on neutrophils and is upregulated on neutrophils in murine cecal ligation and puncture (CLP)-induced sepsis model (20). Its main ligands include CCL3 [macrophage inflammatory protein-1α (MIP-1α)] and CCL5 [regulated on activation normal T-expressed and secreted (RANTES)]. It has already been shown that MIP-1α enhances the protective innate immune response against sepsis by activating macrophages, whereas RANTES triggers the overproduction of proinflammatory cytokines and chemokines, resulting in aggravated injury and mortality following sepsis (13, 23). Whereas production of these chemokines is essential for host defense against bacteria, overproduction of these inflammatory mediators has been shown to play a deleterious role in pathogenesis of sepsis.

Adhesion molecules are also involved in the multistep process of neutrophil recruitment into tissues (26). Neutrophil tethering and rolling is mainly mediated by the selectin family (P- and E-selectin). Selectin mediates rolling to reduce the velocity of circulating neutrophils so that they can detect chemokines that are immobilized on endothelial cells (25). The adhesion of activated neutrophils to endothelial surfaces results from the interaction of neutrophil surface proteins (CD11/CD18) with endothelial cell surface adhesion molecules such as ICAM-1 (CD54) (2).

BX471 is a potent small-molecule nonpeptide CCR1 antagonist in both human and mouse. It has been reported that BX471 displaces the CCR1 ligands, MIP-1α, RANTES, and MCP-3, with high affinity and is a potent functional antagonist inhibiting a number of CCR1-mediated effects including Ca2+ mobilization and leukocyte migration (18). Moreover, BX471 shows a greater than 10,000-fold selectivity for CCR1 compared with 28 different G-protein coupled receptors (18). The aim of the present study was to evaluate the effect of prophylactic and therapeutic treatment with BX471 on CLP-induced sepsis in the mouse and to investigate the underlying mechanisms.

METHODS

Induction of sepsis. All experiments were approved by the animal ethics committee of National University of Singapore and carried out in accordance with established Guiding Principles for Animal Research. The previously described model of CLP-induced sepsis was used (1, 27). Male Swiss albino mice (25–30 g) were lightly anesthetized with the mixture of ketamine and medetomidine [0.75 ml ketamine (100 mg/ml) and 1 ml medetomidine (1 mg/ml) dissolved in

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Table 1. PCR primer sequences, optimal amplification cycles, optimal annealing temperatures, and product sizes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature</th>
<th>Cycles</th>
<th>Product Size, bp</th>
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<tr>
<td>r18S</td>
<td>Sense: 5′-GTAACCCGTTGAAACGGATT-3</td>
<td>59°C</td>
<td>Lung: 23</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-CGATCCATGGCTAGTAGCCG-3</td>
<td></td>
<td>Liver: 26</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Sense: 5′-CAAGTTGAAGCTTTGAGCCTG-3′</td>
<td>60°C</td>
<td>Liver: 26</td>
<td>437</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-TAAGCTGGAAATGCACGGCCG-3′</td>
<td></td>
<td>Liver: 26</td>
<td></td>
</tr>
<tr>
<td>P-selectin</td>
<td>Sense: 5′-TACGAGCTGACGGACCCCG-3′</td>
<td>60°C</td>
<td>Liver: 36</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-GGCTGACCTGAATATTTAGCCG-3′</td>
<td></td>
<td>Liver: 36</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>Sense: 5′-CTGACTTUGATGCACTTCAGG-3′</td>
<td>60°C</td>
<td>Liver: 33</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-TGATTTAAAGCTTGGACCACT-3′</td>
<td></td>
<td>Liver: 36</td>
<td></td>
</tr>
</tbody>
</table>

8.25 ml of distilled water (7.5 ml/kg) under aseptic conditions. After shaving of the abdominal fur and application of a topical disinfectant, a small midline incision was made through the skin and peritoneum of the abdomen to expose the cecum. The cecal appendage was ligated with Silkam 4-0 thread at 3–5 mm below the ileocecal valve. After this, a small amount of stool was squeezed out through both holes. Finally, the abdomen was stitched up with sterile 22-gauge needle distal to the point of ligation. After this, a small midline incision was made through the skin and peritoneum of the abdomen to expose the cecum. The cecal appendage was ligated with Silkam 4-0 thread at 3–5 mm below the ileocecal valve without a cecal ligation and puncture. Bowel was repositioned, and the abdomen was stitched up with sterile 22-gauge needle distal to the point of ligation. After this, a small amount of stool was squeezed out through both holes. Finally, the abdomen was stitched up with sterile peritoneum 5-0 thread. Animals with sham operation underwent the same procedure without cecal ligation and puncture.

BX471 was dissolved in DMSO 20 mg/ml and was administered to mice at a dose of 100 mg/kg subcutaneously either 30 min before or 30 min after the CLP or sham operation. At 24 h after the operation, animals were killed by an intraperitoneal injection of a lethal dose of pentobarbitone. Harvested heparinized blood was centrifuged, and the plasma was removed and stored at 80°C. Samples of lung and liver were fixed in 4% neutral phosphate-buffered formalin then embedded in paraffin wax. Samples of lungs and livers were frozen in liquid nitrogen and stored at −80°C for subsequent measurement of tissue MPO activity. Samples of lungs were also used for chemokines ELISA assay.

MPO estimation. Neutrophil infiltration in lungs and livers was quantitated by measuring tissue MPO activity. Briefly, the tissue samples were homogenized in phosphate buffer 20 mmol/l, pH 7.4, centrifuged at 10,000 g for 10 min at 4°C, and the resulting pellet was resuspended in phosphate buffer 50 mmol/l, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication for 40 s. The sample was then centrifuged at 10,000 g for 5 min at 4°C, and the supernatant was used for the MPO assay. MPO activity was determined as previously described using tetramethylbenzidine (Sigma) as the substrate. This absorbance was corrected by the weight of the tissue samples (fold increase over control).

Morphological examination. Samples of lung and liver were fixed in 4% vol/vol neutral phosphate-buffered formalin and subsequently dehydrated through a graded ethanol series as described before. After impregnation in paraffin wax, tissue samples were sectioned. Liver and lung sections (5 µm) were stained with hematoxylin and eosin and examined by light microscopy with a Carl-Zeiss microscope (objective lens magnification of ×40; eyepiece magnification of ×10).

Chemokine ELISA assay. Chemokine levels in the lung tissue were measured by homogenizing a sample of freshly obtained tissue in 1 ml of phosphate buffer 20 mmol/l, pH 7.4, subjecting it to centrifugation and quantitating chemokines in the resulting supernatant. Supernatants were assayed for chemokines monocyte chemotactic protein-1 (MCP-1), MIP-1α, and RANTES by sandwich ELISA, according to the manufacturer’s instructions (Duoset kit; R&D Systems, Minneapolis, MN). Briefly, primary antibody was aliquoted onto ELISA plates and incubated at room temperature overnight. Samples and standards were added and incubated for 2 h, the plates were washed, and a biotinylated secondary antibody was added and incubated for 2 h. Plates were washed again, and streptavidin bound to horseradish peroxidase was added for 20 min. After a further wash, tetramethylbenzidine was added for color development, and the reaction was terminated with 2 M H2SO4. Absorbance was measured at 450 nm.

RT-PCR. Total RNA from lung and liver was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The concentration of isolated nucleic acids was determined spectrophotometrically by measuring the absorbance at 260 nm, and the integrity was verified by ethidium bromide staining of 18S and 28S rRNA bands on a denaturing agarose gel. All samples were thereafter stored at −80°C until required. RNA (1 µg) was reversely transcribed by using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) at 25°C for 5 min, 42°C for 30 min, followed by 85°C for 5 min. The cDNA was used as a template for PCR amplification by iQ Supermix (Bio-Rad, Hercules, CA). The primer sequences of 18S, P-selectin, E-selectin, and ICAM-1 were as shown in Table 1. The primers were synthesized by Prologio (Singapore). The reaction mixture was first subjected to 95°C for 3 min for the activation of polymerase. This was followed by an optimal cycle of amplifications (Table 1), consisting of 95°C for 30 s, optimal annealing temperature for 30 s and 72°C for 30 s. PCR amplification was carried out in MyCycler (Bio-Rad, Hercules, CA). PCR products were analyzed on 1.5% wt/vol agarose gels containing 0.5 µg/ml ethidium bromide.

Immunohistochemistry. Lung and live samples were isolated and immediately fixed in formalin (buffered at pH 7.4) for 12 h. The fixed tissues were then embedded in paraffin and kept until use. Paraffin sections (5 µm) were dewaxed, hydrated, and washed, and endogenous peroxidase activity was quenched with 0.3% H2O2 for 15 min before being stained. Antigen retrieval was performed by incubating the slides in preheated citrate buffer (pH = 6.0, 95°C) for 15 min. This was followed by 2-h incubation with corresponding primary antibody at optimal dilution (Table 2). After that, the sections were incubated in corresponding peroxidase-conjugated secondary antibody at optimal dilution (Table 2) for 30 min. Finally, the slides were treated with chromogen 3,3′-diaminobenzidine for 1 min, rinsed, counterstained with HRP, horseradish peroxidase.

Table 2. Antibodies and optimal dilution for immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary Antibody</th>
<th>Optimal Dilution</th>
<th>Secondary Antibody</th>
<th>Optimal Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>Rat anti-mouse ICAM-1 monoclonal antibody (Chemicon)</td>
<td>1:300</td>
<td>Goat anti-rat IgG:HRP (Serotec)</td>
<td>1:50</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Goat anti-mouse P-Selectin IgG (Biovision)</td>
<td>1:50</td>
<td>Rabbit anti-goat IgG: Peroxidase (Sigma)</td>
<td>1:50</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Rabbit anti-E-Selectin polyclonal antibody (Biovision)</td>
<td>1:300</td>
<td>Goat anti-rabbit IgG:HRP (Chemicon)</td>
<td>1:100</td>
</tr>
</tbody>
</table>

HRP, horseradish peroxidase.
with hematoxylin, dehydrated in Histoclear, and coverslipped. Negative controls were included by replacing the primary antibody with PBS and showed no immunostaining. All incubations and washes were performed with PBS (3 × 5 min) and carried out at room temperature (25°C), unless otherwise stated. The sections were then examined under a light microscope.

Statistics. Data are expressed as the means ± SE. In all figures, vertical bars denote SE, and absence of such bars indicates that the SE is too small to illustrate. The significance of changes was evaluated by ANOVA when comparing three or more groups. If ANOVA indicated a significant difference, the data were analyzed by Tukey’s method as a post hoc test for the difference between groups. A P value of <0.05 was considered to indicate a significant difference.

RESULTS

Treatment with BX471 attenuated sepsis-induced lung and liver injury. CLP-induced systemic inflammation was indicated by the lung and liver MPO activity, a marker of neutrophil infiltration. As expected, lung MPO activity was significantly increased 24 h after CLP compared with control and sham-operated groups. Similarly, in liver, a significant but smaller elevation in MPO activity was observed 24 h after CLP. Administration of BX471 either 30 min before (prophylactic) or 30 min after (therapeutic) CLP significantly reduced lung and liver MPO levels in animals (Fig. 1). Histology examination showed the morphological changes in the lungs and livers following induction of sepsis. The lung sections from placebo-treated mice with CLP operation exhibited characteristic signs of lung injury, which included interstitial edema, alveolar thickening, and severe leukocyte infiltration in the interstitium and alveoli (compare Fig. 2A to 2B). In liver sections, hepatocyte swelling, slight hepatocyte necrosis, and neutrophil infiltration could be easily observed (compare Fig. 3A to 3B). The protective effect of treatment with BX471 was shown in Fig. 2, C and D and Fig. 3, C and D.

Effect of BX471 on chemokine levels in lung. Treatment with BX471 resulted in upregulation of MIP-1α protein in lung 24 h after CLP (Fig. 4). However, CC chemokine RANTES and MCP-1 expression was not affected by treatment with BX471 (data not shown).

Effect of BX471 on lung and liver ICAM-1, P-selectin, and E-selectin mRNA levels in sepsis. CLP-induced sepsis resulted in significant upregulation on ICAM-1 (Fig. 5, A and F), P-selectin (Fig. 6, A and F), and E-selectin (Fig. 7, A and F) gene expression compared with control group in lung and liver. Treatment with BX471 led to a downregulation of these adhesion molecules at mRNA level compared with placebo-treated groups in lung and liver.

Effect of BX471 on lung and liver ICAM-1, P-selectin, and E-selectin protein expression in sepsis. Protein expression of ICAM1, P-selectin, and E-selectin in lung and liver was investigated by immunohistochemistry. In lungs P-selectin and E-selectin was localized on vascular surface; however, ICAM-1 was detected throughout the alveolar epithelium and endothelial cells (Figs. 5, B–E, 6, B–E, and 7, B–E). In liver ICAM-1, P-selectin, and E-selectin was mainly localized on vascular surface (Figs. 5, G–J, 6, G–J, and 7, G–J). An increase in ICAM-1, P-selectin, and E-selectin protein expression in lung (compare C to B in Figs. 5–7) and liver (compare H to G in Figs. 5–7) during sepsis was observed. Treatment with BX471 either 30 min before or 30 min after CLP procedure led to a downregulation of ICAM-1, P-selectin, and E-selectin protein expression in these organs (D, E, I, and J of Figs. 5–7).

DISCUSSION

In general terms, the systemic inflammatory response is an entirely normal host response to remove pathogens; however, if excessive, it may lead to damage to host tissues of lung and liver systems, multiple organ dysfunction syndrome, and a high mortality rate (3). Because it is well established that chemokines play a key role in controlling leukocyte recruitment and activation, many researchers have investigated the role of chemokine receptors during sepsis (17). Most of these studies have focused on CXC chemokine receptors, especially CXCR1 and CXCR2. Mice deficient in CXCR2 or treated with CXCR2-specific antibodies are protected from developing sepsis (14). Blockade of CXCR2 by antileukinate, a hexapeptide inhibitor of CXC-chemokine receptor, significantly attenuates lung damage (9). Moreover, peptidocins derived from intracellular loops of CXCR1 and CXCR2 reverse the lethal consequence of sepsis, including disseminated intravascular coagulation and multiple organ failure in mice (8).
Recent studies reveal that CCR1 may also play an active role in the progress of sepsis. In the mouse, MIP-1α is a potent mediator for neutrophil recruitment and activation (21). It is also shown that neutrophil CCR1 mRNA expression was upregulated during CLP-induced sepsis (20). The first attempt to study the role of CCR1 in sepsis has shown that CCR1 knockout mice are significantly protected against CLP-induced lethality (13). However, it is possible that the protective effect of CCR1 deficiency in sepsis may be due to its influence on the developmental process of macrophage (5, 12). Therefore, a small-molecule nonpeptide CCR1 antagonist was employed in the present study to demonstrate the role of CCR1 during sepsis.

Although it was reported that CCR1 deficiency had no effect on the inflammatory cell recruitment to the peritoneal cavity, our data have shown that blockage of CCR1 by BX471
attenuates MPO activity in both lung and liver tissues, which is consistent with previous report that blockage of MIP-1α, a main ligand of CCR1, by antibodies attenuates MPO activity in lung in CLP mice, indicating that CCR1 play a key role in controlling neutrophil infiltration into the tissues (13, 20). Because activated neutrophils release protease and reactive oxygen species that lead to host tissue damage during sepsis, downregulation of neutrophils infiltration may contribute to the protective effect in lung and liver in BX471-treated groups as shown in histology examination (16). Our data also show that blocking the chemokine receptor CCR1 by antagonist leads to upregulated expression of MIP-1α in lungs.

In sepsis, endothelial cells can produce cytokines and chemokines and express cellular adhesion molecules in response to an inflammatory stimulus (22). Therefore, we investigate the interaction between CCR1 and cellular adhesion molecules that are expressed on the vascular endothelium. Our results show that blockage of CCR1 by BX471 leads to a downregulation of P-selectin, E-selectin, and ICAM-1 at both mRNA and protein levels in lung and liver tissues. The underlying mechanism is not clear yet. However, it has been observed that challenge with MIP-1α and MCP-1 has no effect on the expression of P-selectin on endothelial cells in vitro, indicating that CC chemokines may not be capable to directly activate endothelial cells (24). Recent studies have suggested that mast cells play an intermediate role in chemokine-induced neutrophil recruitment in vivo by releasing TNF-α to activate endothelial cells.

Fig. 4. Effect of BX471 on chemokine macrophage inflammatory protein-1α (MIP-1α) levels in lung. Values are means (SE) for 8–12 animals in each group. *P < 0.05, #P < 0.01 vs. placebo-treated animals.

Fig. 5. Effect of BX471 on lung and liver ICAM-1 levels in CLP-induced sepsis. A: ICAM-1 expression at mRNA level in lung. B–E: representative immunohistochemical staining of ICAM-1 in the lung tissue sections. B: control, no sepsis. C: CLP-induced sepsis in mice administered placebo 30 min before CLP procedure. D: CLP-induced sepsis in mice administered BX471 30 min before CLP procedure. E: CLP-induced sepsis in mice administered BX471 30 min after CLP procedure. F: ICAM-1 expression at mRNA level in liver. G–J: representative immunohistochemical staining of ICAM-1 in the liver tissue sections. G: control, no sepsis. H: CLP-induced sepsis in mice administered placebo 30 min before CLP procedure. I: CLP-induced sepsis in mice administered BX471 30 min before CLP procedure. J: CLP-induced sepsis in mice administered BX471 30 min after CLP procedure. Values are means (SE) for 6 animals in each group. #P < 0.01 vs. placebo treated animals. Immunohistochemistry figures are representative of at least 3 experiments. Original magnification ×400.
It is evident that other inflammatory cells including macrophages/monocytes, lymphocytes, and other mediators including IL-1β and leukotrienes may also play a key role in this complex interaction between CC chemokines, endothelial cells, and neutrophil recruitment. Downregulation of these adhesion molecules by BX471 may contribute to the attenuation of neutrophil recruitment and activation.

In the present study CCR1 is blocked by a nonpeptide CCR1 antagonist BX471. BX471 has shown a therapeutically effect in several animal models including multiple sclerosis, progressive kidney disease, and organ transplant rejection (18). Moreover, BX471 also has a high affinity for human CCR1. BX471 is now moving from the laboratory bench to the clinic. Phase 2 clinical studies have been initiated to evaluate its safety and efficacy as a therapeutic agent for multiple sclerosis and psoriasis in addition to clinical research to evaluate BX471 as an Alzheimer’s diagnostic targeting CCR1 (6, 7). In the future this antagonist may also be useful to evaluate the therapeutic potential of blocking CCR1 during sepsis in humans.

In summary, we have examined the effect of a CCR1 antagonist BX471 on systemic inflammatory response using a model of sepsis induced by CLP operation. Treatment with BX471 results in significant protection against lung and liver damage, as shown by MPO levels and histology. We also show that blocking CCR1 leads to a downregulation of P-selectin, E-selectin, and ICAM-1 in both lung and liver, suggesting that there is a complex interaction between chemokines and adhesion molecules on endothelial cells in sepsis although the underlying mechanism is not fully understood. Despite more than 20 years of extensive study, sepsis is still the main cause of death in intensive units. Blockage of chemokine receptors by specific antagonist might be a promising strategy for treatment of sepsis (15). It is known that the onset and progression of sepsis to multiple organ dysfunction develops in hours to days in CLP models, whereas this process occurs in days to weeks in human patients. It has been reported that in rat the activation of p38 MAPK is increased in liver 30 min after CLP procedure indicating rapid host response against infection in rodent CLP models (11). At 30 min after CLP procedure downstream pathways have already been activated and administration of BX471 at this time point has shown protective effect in our study.

The present work, therefore, shows that both the prophylactic and therapeutic treatment with CCR1 antagonist BX471 has a protective effect against CLP-induced sepsis.

![Fig. 6. Effect of BX471 on lung and liver P-selectin levels in CLP-induced sepsis. A: P-selectin expression at mRNA level in lung. B–E: representative immunohistochemical staining of P-selectin in the lung tissue sections. B: control, no sepsis. C: CLP-induced sepsis in mice administered placebo 30 min before CLP procedure. D: CLP-induced sepsis in mice administered BX471 30 min before CLP procedure. E: CLP-induced sepsis in mice administered BX471 30 min after CLP procedure. F: P-selectin expression at mRNA level in liver. G–J: representative immunohistochemical staining of P-selectin in the liver tissue sections. G: control, no sepsis. H: CLP-induced sepsis in mice administered placebo 30 min before CLP procedure. I: CLP-induced sepsis in mice administered BX471 30 min before CLP procedure. J: CLP-induced sepsis in mice administered BX471 30 min after CLP procedure. Values are means (SE) for 6 animals in each group. #P < 0.01 vs. placebo treated animals. Immunohistochemistry figures are representative of at least 3 experiments. Original magnification ×400.](G1178)
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GRANTS

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