Murine model of gastrointestinal ischemia associated with complement-dependent injury

HUI ZHAO, MICHAEL C. MONTALTO, KRISTINE J. PFEIFFER, LIMING HAO, AND GREGORY L. STAHL

Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative, and Pain Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115; and Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510

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Gastrointestinal ischemia-reperfusion (I/R) injury is often associated with remote tissue injury. Complement activation plays an important role in local and remote tissue injury associated with gastrointestinal I/R. We developed a new murine model of gastrointestinal I/R that has complement-dependent local and remote tissue injury. Twenty, but not thirty, minutes of gastrointestinal ischemia followed by 3 h of reperfusion induced a significant loss of intestinal lactate dehydrogenase that was significantly prevented by a murine anti-murine C5 monoclonal antibody. Anti-C5 also significantly decreased neutrophil infiltration into the gut and lung. Gastrointestinal I/R significantly increased pulmonary intercellular adhesion molecule-1 mRNA and protein expression that was significantly inhibited by anti-C5. Pulmonary macrophage inflammatory protein-2 mRNA was significantly induced by gastrointestinal I/R and inhibited by anti-C5 treatment. These data demonstrate that brief periods of murine gastrointestinal I/R activate complement, leading to tissue injury and neutrophil accumulation. Anti-C5 treatment attenuates tissue injury, neutrophil recruitment, and leukocyte adherence molecule and chemokine expression in the mouse. This model will be well suited to investigate the role of complement-mediated tissue injury and gene expression after gastrointestinal I/R.

Zhao, Hui, Michael C. Montalto, Kristine J. Pfeiffer, Liming Hao, and Gregory L. Stahl. Murine model of gastrointestinal ischemia associated with complement-dependent injury. J Appl Physiol 93: 338–345, 2002. First published March 29, 2002; 10.1152/japplphysiol.00159.2002.—Gastrointestinal ischemia-reperfusion (I/R) injury is often associated with remote tissue injury. Complement activation plays an important role in local and remote tissue injury associated with gastrointestinal I/R. We developed a new murine model of gastrointestinal I/R that has complement-dependent local and remote tissue injury. Twenty, but not thirty, minutes of gastrointestinal ischemia followed by 3 h of reperfusion induced a significant loss of intestinal lactate dehydrogenase that was significantly prevented by a murine anti-murine C5 monoclonal antibody. Anti-C5 also significantly decreased neutrophil infiltration into the gut and lung. Gastrointestinal I/R significantly increased pulmonary intercellular adhesion molecule-1 mRNA and protein expression that was significantly inhibited by anti-C5. Pulmonary macrophage inflammatory protein-2 mRNA was significantly induced by gastrointestinal I/R and inhibited by anti-C5 treatment. These data demonstrate that brief periods of murine gastrointestinal I/R activate complement, leading to tissue injury and neutrophil accumulation. Anti-C5 treatment attenuates tissue injury, neutrophil recruitment, and leukocyte adherence molecule and chemokine expression in the mouse. This model will be well suited to investigate the role of complement-mediated tissue injury and gene expression after gastrointestinal I/R.

Address for reprint requests and other correspondence: G. L. Stahl, Center for Experimental Therapeutics and Reperfusion Injury, Thorn 705, Brigham and Women’s Hospital, 75 Francis St., Boston, MA 02115 (E-mail: gstahl@zeus.bwh.harvard.edu).

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oped. In this report, we developed a mouse model of gastrointestinal injury that involves complement-mediated injury to the local gastrointestinal tract and complement-dependent pulmonary injury. This model is well suited to evaluate the role of complement-mediated tissue injury in complement-deficient as well as complement knockout mice.

**MATERIALS AND METHODS**

**Intestinal I/R Injury**

Adult male C57B6 mice (Charles River Laboratories), aged 6–8 wk and weighing 20–22 g, were anesthetized with pentobarbital sodium (50 mg/kg ip) and ketamine (50 mg/kg ip). Intestinal ischemia was produced by occlusion of the superior mesenteric artery with surgical, microvascular clips (Roboz, Rockville, MD) for 20 min. Pilot studies revealed that reproducible and consistent data could not be obtained if the ischemic period exceeded 20 min (e.g., death, variations in tissue biochemical endpoints). Reperfusion was achieved by removing the clips; the wound sites were then closed, and mice were kept anesthetized and warm throughout the 3 h of reperfusion. Sham-operated controls underwent the same surgical procedures without clamping. Mice were euthanized by pentobarbital sodium overdose.

**Inhibition of the Terminal Complement Components Using Anti-Mouse C5 MAb**

Murine anti-mouse C5 MAb (BB5.1) is an IgG1 subtype, which was purified from mouse ascites fluid by protein A chromatography. BB5.1 functionally blocks the cleavage of C5 and inhibits the formation of C5a and C5b-9 (25). An isotype-matched control MAb (GS-1) was used as a control (19). Antibodies BB5.1 or GS-1 (50 mg/kg iv) were administered 5 min before the induction of intestinal ischemia.

**Lactate Dehydrogenase Activity in Intestinal Homogenates**

Intestinal lactate dehydrogenase (LDH) activity from tissue homogenates was measured as an index for tissue injury (23). Tissue samples (starting 10 cm above cecum; 15 cm in total length) were collected, cleaned and frozen at −80°C-dianisidine and its loss from the tissue was used as a biochemical marker of cellular injury.

**Myeloperoxidase Activity in Tissue Homogenates**

Myeloperoxidase (MPO) activity, an index of neutrophil infiltration, was measured as described previously (23). The LDH homogenate pellets or lung sections were homogenized in 0.5% hexadecyltrimethyl ammonium bromide (Sigma Chemical) dissolved in 50 mmol/l potassium phosphate buffer (pH 6.0; 10% wt/vol). After homogenates were frozen, thawed, and sonicated three times, the ice-cold samples were centrifuged at 12,500 g at 4°C for 15 min. The supernatants were collected and reacted with 0.167 mg/ml of o-dianisidine dihydrochloride (Sigma Chemical) and 0.0005% H2O2 (Sigma Chemical) in 50 mmol/l potassium phosphate buffer (pH 6.0). The change in absorbance was measured spectrophotometrically at 460 nm every 15 s for 4 min, and the linear portion of the tracing was used for analysis. One unit of MPO activity was defined as the quantity of enzyme that hydrolyzed 1 μmol H2O2/min at 25°C. MPO activity was expressed as units per 100 mg of wet tissue.

**Hematoxylin-Eosin Staining**

Tissue samples for histological staining were taken and fixed in 10% formalin-PBS at 4°C overnight. The samples were dehydrated and embedded in paraffin. Sections (7 μm) were cut and stained with hematoxylin-eosin. Slides were evaluated in a blinded manner.

**Immunohistochemistry**

Purified hamster anti-mouse CD54 (ICAM-1) antibody (BD Pharmingen, San Diego, CA) (1:100) and horseradish peroxidase-conjugated anti-hamster antibody (BD Pharmingen) were used for immunohistochemical staining. A purified hamster IgG (isotype control antibody: anti-keyhole limpet hemocyanin) was used as a negative control.

**Quantitative RT-PCR**

Total RNA was extracted from frozen tissues as previously described with acid guanidinium thiocyanate extraction (4). RNA was incubated with RNase-free DNase (GIBCO Life Technologies, Gaithersburg, MD) at 10 units/100 μg RNA in the presence of 10 mmol/l MgCl2 and 10 units of RNase inhibitor (Boehringer Mannheim, Indianapolis, IN) at 37°C for 20 min. RNA was phenol-chloroform extracted and ethanol precipitated. cDNA was synthesized with random primers using the reverse transcription system from Promega (Madison, WI) according to the manufacturer's instructions. cDNA was amplified in 25-μl reactions containing 1× platinum quantitative PCR SuperMix-UDG buffer (GIBCO Life Technologies) supplemented with 0.133× SYBR green I nucleic acid gel stain (Molecular Probes, Eugene, OR), 1 μl of cDNA reaction mix, and 0.2 μmol/l of each primer. β-Actin, ICAM-1, and macrophage inflammatory protein (MIP)-2 were amplified with an initial soak at 94°C for 10 min followed by 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s for a total of 40 cycles, followed by a 10-min extension at 72°C with the following primers: 5′-gtacagctccaccaacag-3′ and 5′-gtcatagcttcttttcggg-3′ (β-actin), 5′-ggagacgcagaggacct-3′ and 5′-gctcagaagaaccaccttc-3′ (ICAM-1), and 5′-catctcagcctcgc-3′ and 5′-gctggagctgacct-3′ (MIP-2). Amplification and analysis were performed with an iCycler detection system (Bio-Rad). The comparative threshold cycle (CT) method was used to quantify the relative increases (in fold) mRNA levels (28). The average CT value for seven sham-operated controls was used as the calibrator. The efficiency of amplification was confirmed to be near equal for the housekeeping gene and the gene of interest. The value for efficiency was used in the comparative CT equation as described (16). Water samples or RNA samples containing no reverse transcriptase were amplified in parallel to ensure that no contaminating DNA was present during PCR.

**Validation of the Model**

To validate the use of this model and to confirm the importance of the complement component C5, male C5-deficient and their congenic C5-sufficient (control) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice underwent the same experimental conditions as outlined above and were evaluated for biochemical indexes of tissue injury.

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Statistical Analysis

All values are presented as means ± SE of n independent experiments. All data were subjected to one-way ANOVA followed by the Student-Newman-Keul’s post hoc test with the use of SigmaStat software (SPSS Science, Chicago, IL). Differences were considered significant at P < 0.05. A Student’s t-test was used to evaluate the pulmonary MPO or intestinal LDH data for the C5-deficient and C5-sufficient mice.

RESULTS

Assessment of Intestinal Injury

Histology. Representative histological sections from the mouse jejunum in sham-operated mice and I/R mice treated with isotype control MAb or anti-C5 are presented in Fig. 1. We observed a reduction in villi height, focal coagulative necrosis, and acute inflammation in mice treated with the isotype control MAb after 20 min of ischemia and 3 h of reperfusion compared with control sham-operated mice (Fig. 1, B and A, respectively). Ghosts of preexisting villous cores and dead enterocytes were observed. Mice treated with vehicle (i.e., PBS) and undergoing the same period of I/R manifested the same degree of histological and biochemical injury (see below) as control MAb-treated mice (data not presented). Pretreatment with the mouse anti-mouse C5 MAb improved the changes in histology induced by I/R (Fig. 1C). Focal mild villous shortening was observed in anti-C5-treated mice.

LDH activity analysis. We analyzed tissue LDH activity in the intestine of sham-operated mice as well as in isotype control or anti-C5 MAb-treated mice after intestinal I/R as a biochemical index of tissue injury (Fig. 2). Isotype control MAb-treated mice undergoing I/R demonstrated a significant loss of LDH activity compared with sham-operated mice (P < 0.05; Fig. 2). Pretreatment with anti-C5 MAb significantly reduced the loss of LDH activity compared with isotype control

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**Fig. 1.** Hematoxylin and eosin-stained microscopic images of the intestine after ischemia-reperfusion (I/R). Tissue sections (7 μm) are from sham-operated control mice (A) and mice subjected to 20 min of gastrointestinal ischemia and 3 h of reperfusion pretreated with isotype control monoclonal antibody (MAb) (B) or pretreated with anti-C5 (C). Original magnification = ×100. Each micrograph is representative of 3 separate experiments.

**Fig. 2.** Effect of anti-C5 MAb on I/R-induced intestinal injury. Intestinal lactate dehydrogenase (LDH) activity from sham-operated mice or I/R mice pretreated with isotype control MAb or pretreated with anti-C5 were measured (expressed as units/mg protein). Each bar represents mean ± SE from 7 different animals. *P < 0.05 vs. sham-operated or anti-C5-treated groups.
MAb-treated mice. The LDH activity of anti-C5 treated mice undergoing I/R was not significantly different from sham-operated mice (Fig. 1). These data suggest that local intestinal injury after I/R was dependent on C5 cleavage.

MPO activity analysis. The terminal complement components (i.e., C5a and C5b-9) play a key role in the activation, adherence, recruitment, and accumulation of neutrophils into I/R organs (20, 23). The intestinal MPO activity of sham-operated, isotype control-treated, and anti-C5-treated animals undergoing intestinal I/R is presented in Fig. 3. We observed a significant increase in intestinal MPO activity of isotype control MAb-treated mice compared with sham-operated mice ($P < 0.05$). Mice treated with anti-C5 MAb had significantly lower MPO activity than isotype control MAb-treated mice undergoing intestinal I/R ($P < 0.05$). There was no significant difference in intestinal MPO activity between anti-C5-treated mice undergoing I/R and sham-operated mice. These data demonstrated that neutrophil infiltration in the I/R intestine could be significantly attenuated by anti-C5 treatment.

**Assessment of Lung Injury**

**Histology.** A significant component of gastrointestinal I/R injury is the involvement of an inflammatory response in the lung. Pulmonary injury was observed microscopically in sham-operated mice and mice undergoing I/R (Fig. 4). Hematoxylin-eosin staining revealed marked pulmonary congestion and diffuse mild interstitial neutrophilic infiltrates after gastrointestinal I/R in control MAb-treated mice compared with sham-operated controls. Treatment with anti-C5 MAb attenuated the pulmonary congestion and the influx of neutrophils.
inflammatory cells compared with control MAb-treated mice. These data suggest that remote organ injury, secondary to gastrointestinal I/R, is mediated by C5 activation.

MPO activity analysis. MPO activity was used to quantify neutrophil accumulation in the lung. Pulmonary MPO activity was significantly (P < 0.05) increased after gastrointestinal I/R in isotype control MAb-treated mice compared with sham-operated controls (Fig. 5). Pretreatment with anti-C5 significantly inhibited pulmonary MPO activity after gastrointestinal I/R compared with isotype control-treated mice. There was no significant increase in pulmonary MPO activity in anti-C5 MAb-treated mice compared with sham-operated controls. These data suggest that complement activation plays an important role in neutrophil trafficking in the lung after gastrointestinal I/R.

ICAM-1 expression by quantitative RT-PCR. An important component in pulmonary neutrophil trafficking is the expression of leukocyte adhesion molecules (8). ICAM-1 mRNA expression in the lung was investigated with real-time quantitative RT-PCR. We observed a significant (P < 0.05) twofold increase in the ICAM-1 mRNA expression in the murine lung after gastrointestinal I/R relative to sham-operated controls (Fig. 6A). Anti-C5 MAb therapy significantly reduced ICAM-1 mRNA expression compared with isotype control-treated animals. The level of ICAM-1 expression in anti-C5 MAb-treated mice was similar to that in sham-operated controls (Fig. 6A).

ICAM-1 protein expression. The action of anti-C5 MAb treatment on ICAM-1 protein expression in the lung was further examined by immunohistochemistry. Staining with the isotype control antibody (i.e., hamster anti-KLH IgG) revealed no staining in any sections (data not shown). ICAM-1 protein was constitutively expressed in very low amounts in sham-operated controls (Fig. 6B). Isotype control MAb-treated mice had elevated ICAM-1 protein expression in the lung after gastrointestinal I/R compared with sham-operated controls (Fig. 6, C and B, respectively). ICAM-1 protein expression was reduced in the lung of anti-C5-treated mice compared with isotype control-treated mice. The level of ICAM-1 expression in anti-C5 treated mice after gastrointestinal I/R was similar to sham-operated controls (Fig. 6D). These data demonstrate that inhibition of C5 decreases pulmonary ICAM-1 mRNA and protein expression after gastrointestinal I/R.

Regulation of the chemokine, MIP-2. The chemokine, MIP-2, belongs to a superfamily of small proteins that are produced on cellular activation and are involved in leukocyte trafficking and recruitment (17). Pulmonary MIP-2 mRNA expression was significantly (P < 0.05) increased 24 ± 8-fold in mice after gastrointestinal I/R and treatment with isotype control MAb compared with sham-operated controls (Fig. 7). Anti-C5 MAb-treated animals had significantly reduced MIP-2 mRNA expression compared with the control MAb-treated animals. MIP-2 mRNA expression in anti-C5 MAb-treated animals was 6 ± 3-fold greater than sham-operated controls, but this was not statistically significant. These data suggest that in addition to C5a- and C5b-9-mediated neutrophil activation and chemotaxis, MIP-2 may also be involved in pulmonary neutrophil sequestration following gastrointestinal I/R. Furthermore, it suggests that complement activation at the level of C5 is a major contributor to MIP-2 expression in the lung following gastrointestinal I/R.

Validation of the Model

To validate the model and to state conclusively an important role of the complement component in this murine model of gastrointestinal I/R injury, C5-deficient and their congenic control mice were used. We have previously demonstrated that, after kidney ischemia and reperfusion in mice, MAb treatment with BB5.1 in wild-type mice or the use of C5-deficient mice yields similar tissue-protective action (30). As shown in Fig. 8, a significant decrease in pulmonary neutrophil accumulation (e.g., MPO activity) was observed in C5-deficient mice (Fig. 8A). Furthermore, intestinal injury was significantly less in the C5-deficient mice compared with the C5-sufficient controls (Fig. 8B). Thus these data confirm an important role for complement activation and tissue injury in local and remote tissues after gastrointestinal I/R. Furthermore, C5 is an important contributor to the local and remote tissue injury.

DISCUSSION

Gastrointestinal I/R is a common clinical problem caused by many medical conditions, including acute mesenteric ischemia, small bowel transplantation, abdominal aortic aneurysm, hemorrhagic shock, necrotizing enterocolitis, septic shock, and severe burns (5, 10–12, 21). Gastrointestinal I/R is often associated with the development of systemic inflammation, pul-

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monary microvascular dysfunction, and adult respiratory distress syndrome (5, 9, 10). Effective therapeutics to eliminate or attenuate local and remote tissue injury after gastrointestinal I/R are needed. Our laboratory has recently demonstrated that complement activation in the rat after gastrointestinal I/R mediates a substantial portion of the local tissue injury and identified several key proinflammatory genes that were regulated by complement C5 activation in the gut (23). In that report, we also observed significant pulmonary injury secondary to the gastrointestinal I/R. However, the potential molecular mechanisms of the pulmonary injury were not investigated in our rat model. The present study was developed to extend our previous findings in the rat, to investigate the pulmonary injury component after gastrointestinal I/R, and to establish a mouse model of complement-dependent local and remote organ injury after gastrointestinal I/R.

Our mouse model of gastrointestinal I/R (i.e., 20 min of ischemia and 3 h of reperfusion) led to local and remote tissue injury that was significantly attenuated by anti-C5 MAb. Furthermore, we extend and validate

Fig. 6. Pulmonary intercellular adhesion molecule-1 (ICAM-1) expression after gastrointestinal I/R. A: ICAM-1 mRNA expression. Quantitative RT-PCR for ICAM-1 mRNA was performed and expressed as the relative fold increase in ICAM-1 mRNA from sham-operated mice, I/R mice pretreated with isotype control MAb, or I/R mice pretreated with anti-C5. Gastrointestinal I/R-induced pulmonary ICAM-1 mRNA expression was attenuated with anti-C5 MAb treatment. Each bar represents mean ± SE from 6–7 animals. *P < 0.05 vs. sham or anti-C5 treated groups. Bottom: immunohistochemical staining of pulmonary ICAM-1 after gastrointestinal I/R. Tissue sections from sham-operated mice (B), I/R mice pretreated with isotype control MAb (C), or I/R mice pretreated with anti-C5 (D) were stained with anti-ICAM-1. Gastrointestinal I/R-induced pulmonary ICAM-1 protein expression was attenuated with anti-C5 MAb treatment. Original magnification = ×200. Each micrograph is representative of 3 separate experiments.

Fig. 7. Pulmonary chemokine [macrophage inflammatory protein-2 (MIP-2)] mRNA expression after gastrointestinal I/R. Quantitative RT-PCR results for pulmonary MIP-2 mRNA expression from sham-operated mice, I/R mice pretreated with isotype control MAb, or I/R mice pretreated with anti-C5 are shown. Average value of sham-operated animal was used as the calibrator. Gastrointestinal I/R-induced pulmonary MIP-2 mRNA expression was attenuated with anti-C5 MAb treatment. Each bar represents mean ± SE from 5–7 animals. *P < 0.05 vs. sham-operated or anti-C5-treated groups.
these findings by using the C5-deficient and the congenic control mice. Pulmonary neutrophil accumulation and intestinal damage (e.g., LDH activity) are shown to be significantly less in the C5-deficient mice after gastrointestinal I/R, thus validating the role of C5 in our mouse model. Furthermore, our laboratory previously demonstrated similar protective actions of either anti-C5 MAb or C5-deficient mice in a mouse model of renal ischemia and reperfusion (30). Previous models of gastrointestinal I/R in the mouse demonstrated that local intestinal injury was mediated by IgM, C5b-9, and classical complement pathway activation and was independent of neutrophils (1, 27). Our neutrophil data are at odds with these previous findings. We demonstrate that anti-C5, which inhibits formation of C5a and C5b-9, inhibits neutrophil infiltration into the gut as well as the lung after gastrointestinal I/R (Fig. 3). Furthermore, we also show that intestinal injury or pulmonary neutrophil infiltration is significantly less in the C5-deficient mice compared with their congenic controls (Fig. 8). The reason for the differences observed in our study and the studies in the literature may relate to the length of the ischemic period (e.g., 40 vs. 20 min in our study) (1). Furthermore, our laboratory demonstrated previously in the rat that inhibition of C5 attenuates neutrophil infiltration into the lung and gut after gastrointestinal I/R (23). Pilot studies used to develop the present model (data not presented) demonstrated significantly less protective actions of anti-C5 in mice subjected to ischemic periods greater than 20 min. However, it is clear from these studies that the terminal complement components mediate local and remote tissue injury following gastrointestinal I/R injury.

Our laboratory previously showed that the inhibition of the late complement components attenuates neutrophil accumulation and preserves tissue integrity in the intestine and lung in a rat model of gastrointestinal I/R (23). In the present study, we extend our previous findings to a new mouse model of gastrointestinal I/R and investigate the potential molecular mechanisms regulating pulmonary neutrophil trafficking into the lung. C5a, C5b-7, and C5b-9 are known to have profound influences on neutrophil activation and adherence molecule expression and appear to be the dominant players in vivo during acute inflammation (2, 6, 24). We demonstrate that anti-C5 significantly attenuates the expression of pulmonary ICAM-1 mRNA and protein expression in our model. Along with this finding, we also observe a significant attenuation of neutrophil sequestration within the lung. The chemokine, MIP-2, plays an important role in neutrophil accumulation (6). In addition, bronchoalveolar lavage MIP-2 protein levels during complement activation are decreased by anti-tumor necrosis factor-α (TNF-α) and/or anti-C5a treatment (7). In the present study, we demonstrate that pulmonary MIP-2 expression is significantly upregulated after gastrointestinal I/R. Furthermore, inhibition of C5 significantly decreased MIP-2 mRNA expression to sham-operated control levels. Because combined C5a and TNF-α expression can lead to increased MIP-2 expression (7) and anti-C5 inhibits TNF-α expression (e.g., mRNA and protein) in a rat model of gastrointestinal I/R (23), it is possible that the decreased pulmonary MIP-2 expression in our murine model is a result of decreased TNF-α and C5 activation. It is possible that neutrophil accumulation in our model involved not only C5a generation but also MIP-2 expression. These data demonstrate that C5 inhibition significantly inhibited MIP-2 expression, as well as pulmonary neutrophil accumulation in the lung after gastrointestinal I/R. Our data confirm that inhibition of the terminal complement components prevents local and remote pulmonary neutrophil accumulation and the resulting I/R injury.

In summary, we developed a murine model of gastrointestinal I/R that induces local and remote organ injury that is mediated in part by complement activation. Anti-C5 attenuates gastrointestinal and lung injury after gastrointestinal I/R. Anti-C5 decreased the pulmonary expression of ICAM-1 and MIP-2. These results, as well as others in the literature, clearly demonstrate that inhibition of the terminal complement components leads to decreased expression of multiple proinflammatory genes in many different organs and protects local and remote tissue from I/R-mediated injury (20, 23, 30). This new mouse model coupled with the newly developed knockout animals will allow investigation into the importance of various complement pathways involved in local and remote tissues after gastrointestinal I/R.

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COMPLEMENT AND LUNG INJURY

REFERENCES


