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RESEARCH ARTICLE | *Vascular Biology and Microcirculation*

Myeloperoxidase instigates proinflammatory responses in a cecal ligation and puncture rat model of sepsis

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Yu H, Liu Y, Wang M, Restrepo RJ, Wang D, Kalogeris TJ, Neumann WL, Ford DA, Korthuis RJ. Myeloperoxidase instigates proinflammatory responses in a cecal ligation and puncture rat model of sepsis. *Am J Physiol Heart Circ Physiol* 319: H705–H721, 2020. First published August 7, 2020; doi:10.1152/ajpheart.00440.2020.—Myeloperoxidase (MPO)-derived hypochlorous (HOCl) reacts with membrane plasmalogens to yield α-chlorofatty aldehydes such as 2-chlorofatty aldehyde (2-ClFALD) and its metabolite 2-chlorofatty acid (2-ClFA). Recent studies showed that 2-ClFALD and 2-ClFA serve as mediators of the inflammatory responses to sepsis by as yet unknown mechanisms. Since no scavenger for chlorinated lipids is available and on the basis of the well-established role of the MPO/HOCl/chlorinated lipid axis in inflammatory responses, we hypothesized that treatment with MPO inhibitors (*N*-acetyl lysyltyrosylcysteine amide or 4-aminobenzoic acid hydrazide) would inhibit inflammation and proinflammatory mediator expression induced by cecal ligation and puncture (CLP). We used intravital microscopy to quantify in vivo inflammatory responses in Sham and CLP rats with or without MPO inhibition. Small intestines, mesenteries, and lungs were collected to assess changes in MPO-positive staining and lung injury, respectively, as well as free 2-ClFA and proinflammatory mediators levels. CLP caused neutrophil infiltration, 2-ClFA generation, acute lung injury, leukocyte-/platelet-endothelium interactions, mast cell activation (MCA), plasminogen activator inhibitor-1 (PAI-1) production, and the expression of several cytokines, chemokines, and vascular endothelial growth factor, changes that were reduced by MPO inhibition. Pretreatment with a PAI-1 inhibitor or MC stabilizer prevented CLP-induced leukocyte-endothelium interactions and MCA, and abrogated exogenous 2-ClFALD-induced inflammatory responses. Thus, we provide evidence that MPO instigates these inflammatory changes in CLP and that chlorinated lipids may serve as a mechanistic link between the enzymatic activity of MPO and PAI-1- and mast cell-dependent adhesive interactions, providing a rationale for new therapeutic interventions in sepsis.

NEW & NOTEWORTHY Using two distinct myeloperoxidase (MPO) inhibitors, we show for the first time that MPO plays an important role in producing increases in free 2-chlorofatty aldehyde (2-ClFALD)—a powerful proinflammatory chlorinated lipid in plasma and intestine—a number of cytokines and other inflammatory mediators, leukocyte and platelet rolling and adhesion in postcapillary venules, and lung injury in a cecal ligation and puncture model of sepsis. In addition, the use of a plasminogen activator inhibitor-1 (PAI-1) inhibitor or a mast cell stabilizer prevented inflammatory responses in CLP-induced sepsis. PAI-1 inhibition also prevented the proinflammatory responses to exogenous 2-ClFALD superfusion. Thus, our study provides some of the first evidence that MPO-derived free 2-ClFA plays an important role in CLP-induced sepsis by a PAI-1- and mast cell-dependent mechanism.

2-chlorofatty acid; mast cell activation; myeloperoxidase; plasminogen activator inhibitor-1; proinflammatory mediators

INTRODUCTION

Sepsis is associated with a wide variety of intense inflammatory responses in the microcirculation that include proinflammatory mediator release, marked increases in adhesion molecule expression, overexuberant production of reactive oxygen species (ROS), leukocyte- and platelet-endothelial cell adhesive interactions, mast cell activation, and endothelial barrier disruption (14, 34, 35). Neutrophils are the initial responder in sepsis, and once activated, release their granule contents, especially myeloperoxidase (MPO). In consideration that MPO synthesizes hypochlorous acid (HOCl) and other reactive oxygen species (ROS), neutrophils pose a threat to native cells, causing collateral damage (43, 87). In addition, in the setting of sepsis, the increases in numerous cytokines, chemokines, and some growth factors act to increase adhesion molecule expression on endothelial cells and leukocytes, to promote leukocytes, especially neutrophils to migrate into the tissues.

Although it is well established that MPO-derived HOCl targets membrane plasmalogens in neighboring endothelial cells, leading to the production of 2-chlorofatty aldehyde (2-ClFALD) and its metabolite 2-chlorofatty acid (2-ClFA), their role in diseases characterized by extensive inflammation was not appreciated until recent years (77, 85, 86). However, it is now known that plasma 2-ClFA levels correlate with the appearance and severity of acute respiratory disease syndrome (ARDS) in septic subjects, which led to the notion that plasma 2-ClFA levels may serve as a predictor for the appearance of ARDS. In addition, direct application of 2-ClFALD and 2-ClFA to normal tissues in lieu of sepsis induced inflammatory responses that were very similar to that which occurs in sepsis (53, 91). Identifying a direct role for 2-ClFA in sepsis has proven difficult because there are no scavengers available for chlorinated lipid species generated from MPO/HOCl sys-

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tem in neutrophils (23, 62–64). However, the existence of mechanistically distinct inhibitors of MPO provides a tool to determine whether the catalytic activity of this enzyme contributes to the pathogenesis of inflammation in sepsis. In addition, we used a PAI-1 inhibitor and mast cell stabilizer to evaluate the role of these inflammatory mediators in MPO-induced proinflammatory responses to CLP. By correlating protective actions of these inhibitors on sepsis induced 2-ClFA levels and inflammation, we can begin to explore this link.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri. Sprague-Dawley rats (10–14 wk) acquired from ENVIGO (Indianapolis, IN) were housed in climate-controlled, 12-h:12-h light-dark cycle animal care facilities of the University of Missouri and had ad libitum access to food and water. Upon arrival, rats were housed for at least 7 days before experiments were performed. Rats were selected as the animal model because their neutrophils express MPO at levels that approach those noted in humans, whereas mice MPO express at much lower levels (11, 66, 84).

MPO inhibitors pretreatment. Rats were randomly assigned to the following groups: Sham-operated control (no cecal ligation and puncture, SCLP), CLP alone, induction of CLP in animals pretreated with reversible MPO inhibitor *N*-acetyl lysyltyrosylcysteine amide (KYC, 6 mg/kg; kindly provided by Saint Louis University) 24 h (first dose) and 1 h (second dose) before CLP (92), or pretreated with an irreversible MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH, 40 mg/kg; Sigma-Aldrich, St. Louis, MO) 3 h before CLP surgery, as previously described (24, 67). Two groups of sham-operated control rats were also treated with KYC (SCLP + KYC) or ABAH (SCLP + ABAH).

PAI-039 and cromolyn sodium pretreatment. First dose of PAI-1 inhibitor tiplaxtinin (PAI-039, 44 μg/kg; kindly provided by Dr. William P. Fay) was given by intraperitoneal injection 1 h before surgery for CLP and SCLP groups, followed by treatment with a second dose 1 h before data collection (32). In two additional groups, cromolyn sodium (50 mg/kg) was applied intraperitoneally 30 min before sham or CLP surgery, followed by a second dose at 30 min before the measurements (33).

Lipid superfusion. 2-ClFALD was kindly provided by Dr. David A. Ford from the Center for Cardiovascular Research, Saint Louis University. Experimental group rats were pretreated with PAI-039 (44 μg/kg ip) 1 h before the initiation of 2-ClFALD superfusion. These rats were then anesthetized (detailed description in *CLP-induced sepsis model*), followed by exteriorization of the mesentery, which was then superfused with 2-ClFALD (10 μM) for 90 min. In two other groups, without any pretreatment, exteriorized mesenteries were superfused with nonchlorinated fatty aldehyde (FALD) and 2-ClFALD at the same molar concentration as negative and positive control, respectively. We then evaluated leukocyte rolling at baseline and after 30, 60, and 90 min of superfusion with the lipids described above, and mast cell activation was quantified at the end time point of superfusion.

CLP-induced sepsis model. Rats were anesthetized by intraperitoneal injection of a mixture of ketamine (90 mg/kg body wt dosing) and xylazine (10 mg/kg body wt). Once a deep plane of anesthesia was achieved, the abdomen was shaved, followed by producing a longitudinal abdominal midline incision (3 to 4 cm) to allow visualization of the cecum. After careful dissection of the mesentery of the cecum, a ligature was placed around the cecum at the designated position for the desired severity grade and ligated to occlude the cecal lumen at that point. Cecum was perforated and after removal of the needle, a droplet of feces was extruded to ensure puncture patency. In a sham surgery group, the cecum was exteriorized without ligation or penetration. Upon closure of the abdominal wound with suture, animals were resuscitated and monitored before experimental measurements were obtained via intravital microscopy 6 h after CLP or sham surgery (68). At the end of experiments, tissue samples from the small intestine, mesentery, lung, as well as plasma, were collected for further analysis.

Our experiments were carried out in a modified cecal ligation and puncture model, in which the blood supply for cecum was not fully occluded by placing an 18-gauge needle along the cecum during ligation, which was subsequently withdrawn to produce a calibrated stenosis of the cecal lumen. The advantages of this approach are that 100% of animals remain viable for at least 6 h after this modified CLP surgery. With reduced degree of injury using this modified CLP surgery, mesenteric microvascular patency is preserved, enabling in vivo investigation of microcirculatory responses with intravital microscopy.

Intravital microscopy. Rats were placed on a plexiglas animal board, and the ileocecal portion of the mesentery was exteriorized and draped over a glass coverslip and superfused with prewarmed saline (37°C). The Plexiglas animal board was then mounted on the stage of an inverted microscope (Eclipse TE2000; Nikon) and inflammatory events occurring in mesenteric postcapillary venules and the surrounding mesentery were observed, typically with ×200 magnification (96). Fluorescent images (excitation, 420–490 nm; emission 520 nm) were detected with a charge-coupled device (CCD) camera (Photometrics COOLSNAP ES). Images were projected onto a television monitor (PVM-1953 MD; Sony) and recorded on a DVD recorder (DMR-E50; Panasonic) or captured through Metamorph software version 7.8. A time-date generator (WJ810, Panasonic) displayed time information on the monitor.

Leukocyte-/platelet-endothelium interactions. The procedures and fluorescent labeling of leukocytes and platelets are similar to what we have used previously in our laboratory (13, 96). First, intravenous cannulation was performed along the left jugular vein, followed by carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Molecular Probes, Eugene, OR) injection and mesentery scanning via intravital microscopy. For each rat, 10 single-unbranched postcapillary venules (20–50 μm in diameter and 100 μm in length) in mesentery were monitored. Cell interactions (number of rolling and firmly attached leukocytes and platelets) will be quantified.

Mast cell activation. Ruthenium red (EMD Millipore, St. Charles, MO) was dissolved into borate-buffered saline (BBS) at the concentration of 0.001%, and the uptake of ruthenium red was used as an indicator of mast cell activation (76). Exteriorized mesentery was superfused with ruthenium red (0.001%) for 15 min, after which 10 fields were randomly chosen for quantifying the number of activated mast cells in each field.

Immunohistochemistry staining for MPO in the small intestine. The collected small intestine samples were fixed in 10% (wt/vol) PBS-buffered formaldehyde and embedded in paraffin. Following dewaxing, endogenous peroxidase was quenched with 3% (vol/vol) hydrogen peroxide for 5 min. The slides were incubated in 5% (vol/vol) bovine serum for 20 min to block nonspecific binding. Sections were then incubated with anti-MPO antibody (ab9535, 1:50 in PBS, Abcam, MA) for 60 min. Samples were rinsed in wash buffer and incubated with detection system for 30 min. Slides were applied with 3, 3′-diaminobenzidine (DAB) substrate and stained with Mayer's hematoxylin for 30 s, then dehydrated in graded alcohol and xylene. Immunohistochemical images were collected and for graphic display of staining analysis, the immune reactivity was expressed as the mean total number of positive staining cells in each filed.

Lung wet/dry weight ratio. The lung wet-to-dry weight ratio was obtained after the cecal ligation and puncture surgery as an indicator of lung water accumulation/pulmonary edema. The lungs were dissected 6 h after surgery and when the animals were still under deep anesthesia; then the lung weight was measured immediately after the excision (wet weight). The lung tissue was kept in an oven at 60°C,

and the weight was measured daily until there was no decrease in two consecutive days (dry weight). The wet/dry weight ratio was determined by dividing the wet weight by the dry weight.

Albumin leakage. FITC-labeled albumin (Sigma-Aldrich, St. Louis, MO) was used to evaluate the albumin leakage across postcapillary venules in rat mesenteric circulation. To quantify albumin leakage across mesenteric venules, 50 mg/kg of labeled albumin was administered intravenously to the animals 15 min before the recording. Rat mesentery was exteriorized and prepared for intravital microscopy as described above. Six hours after surgery, 10 single unbranched postcapillary venules (20–50 μm in diameter and 100 μm in length) were selected. Images were captured and analyzed by MetaMorph (Nashville, TN), and the fluorescence intensity of FITC-albumin in five regions of interest (25 μm in diameter) within the venules (intravenously) and in the perivenular interstitium within 10–50 μm of the venular wall (intraperitoneally) was measured using Metamorph software version 7.8. Albumin leakage, estimated intraperitoneally/intravenously at each pair of corresponding circles was designated as the indicator of albumin leakage (96). This method assumes that fluorescent-tagged albumin will move from the microvessel into the tissue space in a manner that reflects the behavior of native albumin.

Histological evaluation of CLP-induced lung injury. Following the dissection of trachea and lungs, a short cross-sectional incision was made in the upper part of the trachea. An 18G blunted needle connected to a 3–5 mL syringe containing 10% formalin was gently threaded into the trachea and then ligated to hold the needle in place. The lungs were gently inflated with formalin until full expansion to a normal level as expected to fill the chest cavity. Ligation of the trachea was performed to keep the lung expanded, and then the organ mass, including all lung lobes and trachea, was placed in formalin for 48–72 h (54). Then the lung was sectioned, placed on glass slides, and stained with hematoxylin-and-eosin. The degree of lung injury was examined, and the specimens were graded by acute lung injury (ALI) score based on *1*) alveolar capillary congestion, *2*) intra-alveolar hemorrhage, *3*) infiltration or aggregation of neutrophils in the airspace or the vessel wall, and *4*) thickness of the alveolar wall/hyaline membrane formation. Each item was graded according to the following five-point scale: 0, minimal damage; 1, mild damage (0–25%); 2, moderate damage (25–50%); 3, severe damage (50–75%); and 4, maximal damage (75–100%) (31). The degree of ALI was assessed by the sum of scores for items 0 to 16 in 10 randomly selected high-power fields (HPFs, ×400 magnification). The average of the sum of each field score was compared among groups (41).

Plasma and mesentery preparation. Using a syringe coated with 0.5 M sterile EDTA, we collected blood from the exposed inferior vena cava via a needle attached to the syringe. After transferring the collected blood into 10-ml EDTA-coated tubes (BD Vacutainer tubes; Thermo Fisher Scientific, Hampton, NH), they were centrifuged at 1,000 *g* for 10 min at 4°C within 30 min of blood collection (Sorvall ST 40R Centrifuge, Thermo Fisher Scientific, Hampton, NH). Collected plasma was immediately decanted and aliquoted for lipid extraction analysis, diluted twofold using 1× PBS at pH ~7.4 for Multiplex analysis, and then stored at −80°C.

Once the mesentery tissues were homogenized and sonicated, they were centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was gathered. Immediately thereafter, total protein amount was determined (Spectophotometer NanoDrop, Thermo Fisher Scientific, Hampton, NH), and samples were normalized to contain an equal amount of protein by adding appropriate volumes of buffer to those samples with higher total protein content. The samples were then stored at −80°C until analysis.

Lipid extraction and analyses. Frozen small intestine samples were pulverized at the temperature of liquid N_2, and free 2-ClFA was extracted from 10 to 50 mg of homogenized tissue with 103 fmol 2-Cl-[d_4]-hexadecanoic acid, as previously described (85). Similarly, free 2-ClFAs in plasma were analyzed by spiking 25 μL of plasma with 103 fmol 2-Cl-[d_4]-hexadecanoic acid, and a modified Dole extraction was performed (17, 85). Lipids isolated from the heptane layer were dried and subsequently suspended in 260 μL of 85/15 methanol/water with 0.1% formic acid. 2-ClFA analysis was performed by LC/MS using electrospray ionization (ESI) and selected reaction monitoring following previously described methods on the triple quadruple mass spectrometer (23, 85). LC was performed using monolithic C18 column, using solvent gradients previously described (23, 85).

Multiplex analysis of PAI-1 and cytokines. Plasma were 2× diluted in 1× PBS solution. The multiplexing analysis was performed using the Luminex 100 system (Luminex, Austin, TX) by Eve Technologies, Corp. (Calgary, Alberta, Canada). Total plasminogen activator inhibitor-1 (tPAI-1) was measured in the samples using a MILLIPLEX rat vascular injury panel 1, 4-plex kit (Millipore), according to the manufacturer's protocol. The assay sensitivities of these markers range from 0.086 to 1.928 ng/mL for the 4-plex. Individual analyte values are available in the MILLIPLEX protocol.

Luminex xMAP technology was also applied for multiplexed quantification of 27 rat cytokines, chemokines, and growth factors in plasma and mesentery, respectively. Twenty-seven markers were simultaneously measured in the samples using a MILLIPLEX rat cytokine/chemokine 27-plex kit (Millipore), according to the standard protocol from manufacturer. The 27-plex consisted of G-CSF, eotaxin, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-1α, leptin, MIP-1α, IL-4, IL-1β, IL-2, IL-6, EGF, IL-13, IL-10, IL-12 (p70), IFNγ, IL-5, IL-17A, IL-18, MCP-1, IP-10, GRO/KC, VEGF, fractalkine, LIX, MIP-2, TNF-α, and RANTES. The assay sensitivities of these markers range from 0.3 to 30.7 pg/mL.

Data analysis and statistics. On the basis of normality tests, multiple comparisons were performed by using one-way ANOVA with Fisher's least significant difference (LSD) test as a post hoc test, or Brown-Forsythe and Welch ANOVA tests. The nonparametric Kruskal-Wallis test was applied to the lung damage scores and mesenteric GM-CSF data. Data were analyzed by the Brown-Forsythe and Welch ANOVA tests, as well as the Kruskal-Wallis test, as specifically indicated in figure legends. One-way ANOVA with Fisher's LSD test was applied to the rest of the data. GraphPad Prism 6.0 was used to generate all statistical values. All data were shown as sample means ± SE. A P value of 0.05 was considered statistically significant, and the error bars represent SE in all figures.

RESULTS

MPO expression and MPO-derived 2-ClFA. In the first series of experiments, we quantified neutrophil infiltration by assessing MPO expression using an immunobiological approach that we employed in an earlier work (91). To determine whether MPO inhibition would limit neutrophil infiltration induced by CLP, some groups were treated with the MPO inhibitors KYC or ABAH. Representative images of MPO staining in SCLP, CLP, SCLP + KYC, CLP + KYC, SCLP + ABAH, and CLP + ABAH are shown in Fig. 1*A*. Quantification (Fig. 1*B*; $P < 0.01$) showed that a significant increase in the number of MPO-positive cells (15.45 ± 0.93; $n = 6$) was induced by CLP challenge compared with the sham surgery (4.97 ± 0.37; $n = 6$). This CLP-induced increase was prevented by pretreatment with KYC (4.97 ± 0.67; $n = 6$) or ABAH (6.87 ± 0.53; $n = 7$), while little effect of these inhibitors was noted in SCLP (SCLP + KYC: 4.91 ± 0.59; $n = 6$; SCLP + ABAH: 5.51 ± 0.55; $n = 8$).

Although a number of studies have provided evidence that indicates MPO is an indicator of inflammation in sepsis (44, 73), including our present and previously published works, the mechanisms whereby MPO may contribute to the proinflammatory effects of sepsis remain unclear. However, it has been

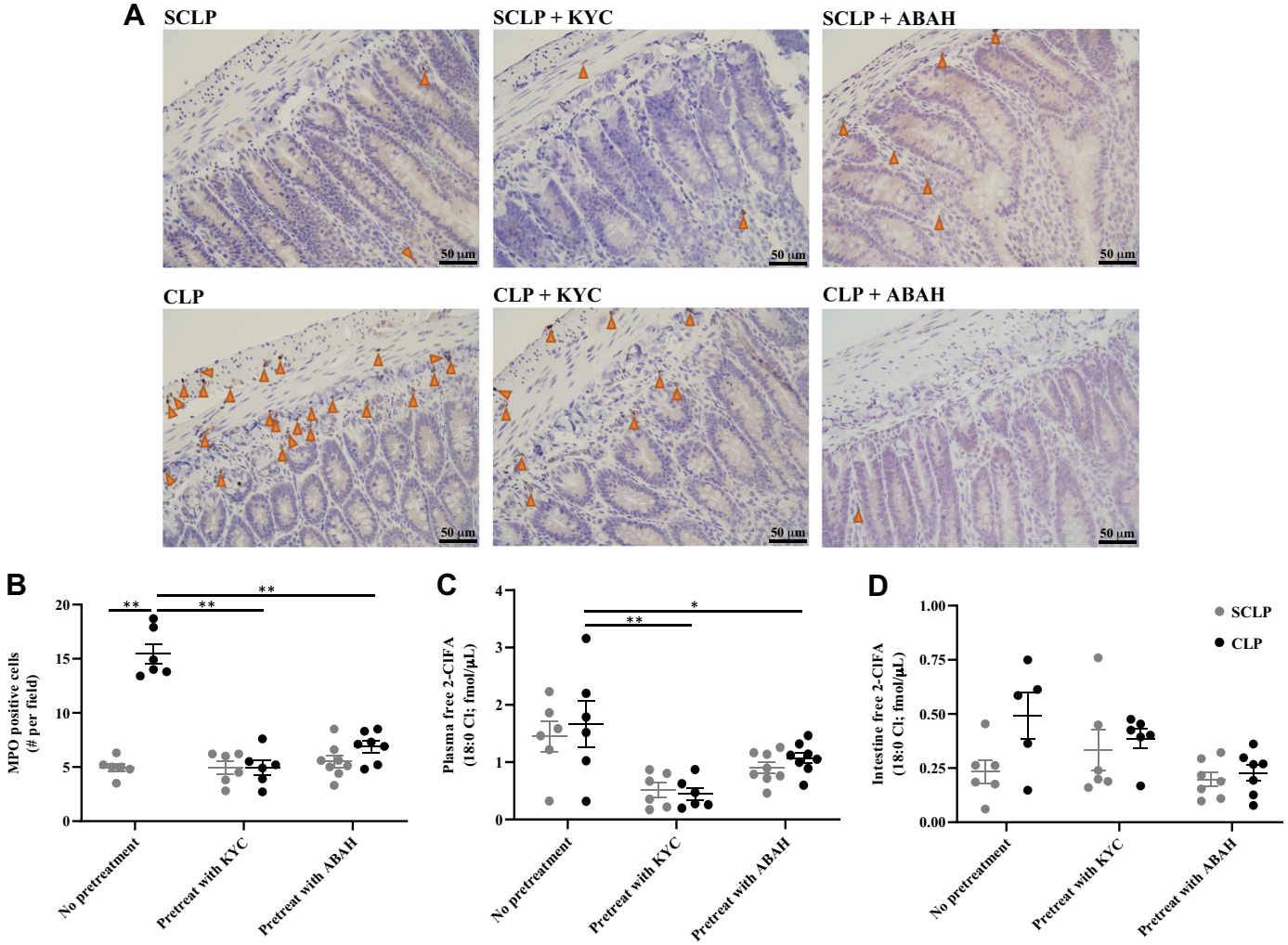

Fig. 1. Myeloperoxidase (MPO) expression in small intestine and associated free 2-chlorofatty aldehyde (2-ClFA) generation. *A*: representative IHC images showing MPO staining is markedly elevated in animals subjected to cecal ligation and puncture (CLP) vs. sham-operated cecal ligation and puncture (SCLP). The orange arrowheads indicate positive staining (brown) cells. Scale bars = 50 μm. *B*: we present the number of MPO-positive cells obtained from 10 fields at ×400 magnification. A significant increase in the number of MPO-positive cells was induced by CLP challenge compared with the sham surgery. This CLP-induced increase was prevented by pretreatment with *N*-acetyl lysyltyrosylcysteine amide (KYC) or 4-aminobenzoic acid hydrazide (ABAH) (CLP + ABAH; $n = 7$ rats), while little effect of these inhibitors was noted in SCLP with MPO inhibitors (SCLP + ABAH; $n = 8$ rats). *C*: CLP induced a modest increase of 2-ClFA in plasma relative to the SCLP group. KYC pretreatment was associated with significantly decreased plasma free 2-ClFA levels in both SCLP + KYC group and CLP + KYC group. As expected, inhibition of MPO with ABAH also dramatically suppressed 2-ClFA production in plasma for SCLP + ABAH ($n = 8$ rats) and CLP + ABAH ($n = 8$ rats). *D*: similarly, compared with SCLP ($n = 5$), CLP provoked an increase of 2-ClFA in small intestine samples, an effect that was largely abolished by treatment with MPO inhibitors before surgery. In ABAH-pretreated groups, $n = 7$ rats. Data are expressed as means ± SE; $n = 6$ unless otherwise stated. *$P < 0.05$ and **$P < 0.01$. Brown-Forsythe and Welch ANOVA tests were used for the data analysis in *D* because the data were not normally distributed.

suggested that MPO-derived 2-ClFA may play a role in human sepsis mortality via induction of ARDS (53). To directly address this question, we evaluated the effect of MPO on the generation of free 2-ClFA in plasma and small intestine obtained from septic rats that were treated with the MPO inhibitors KYC or ABAH. Of note, the molecular species of 2-ClFA that we analyzed is 2-ClFA molecular species, 2-chlorostearic acid (18:0 Cl). As shown in Fig. 1*C*, CLP induced an increase in free 2-ClFA (18:0 Cl) formation, an effect that was reduced by the application of KYC or ABAH, in which KYC reduced free 2-ClFA (18:0 Cl) generation by ~65–75%, whereas ABAH pretreatment lowered CLP-induced increases in 2-ClFA by ~40–45%. We also showed free 2-ClFA levels were increased locally by measuring these levels in samples of small intestine after CLP, effects that were largely abolished by administration of MPO inhibitors before surgery (Fig. 1*D*).

CLP-induced lung injury. As shown in Fig. 2, treatment with the MPO inhibitor KYC reduced CLP-induced pulmonary edema (Fig. 2*A*) and mesenteric venular protein leakage (Fig. 2*B*), which are the hallmarks of systemic inflammatory response syndrome. We next postulated that MPO engages in CLP-induced ARDS mediated by neutrophil activation. Figure 2*C* presents representative histologic images of acute lung injury induced by CLP versus SCLP in the absence and presence of KYC or ABAH. Prominent histologic evidence of lung injury (alveolar congestion, intra-alveolar hemorrhage, neutrophil infiltration, and increased thickness of the alveolar wall) is apparent 6 h after induction of CLP and was largely

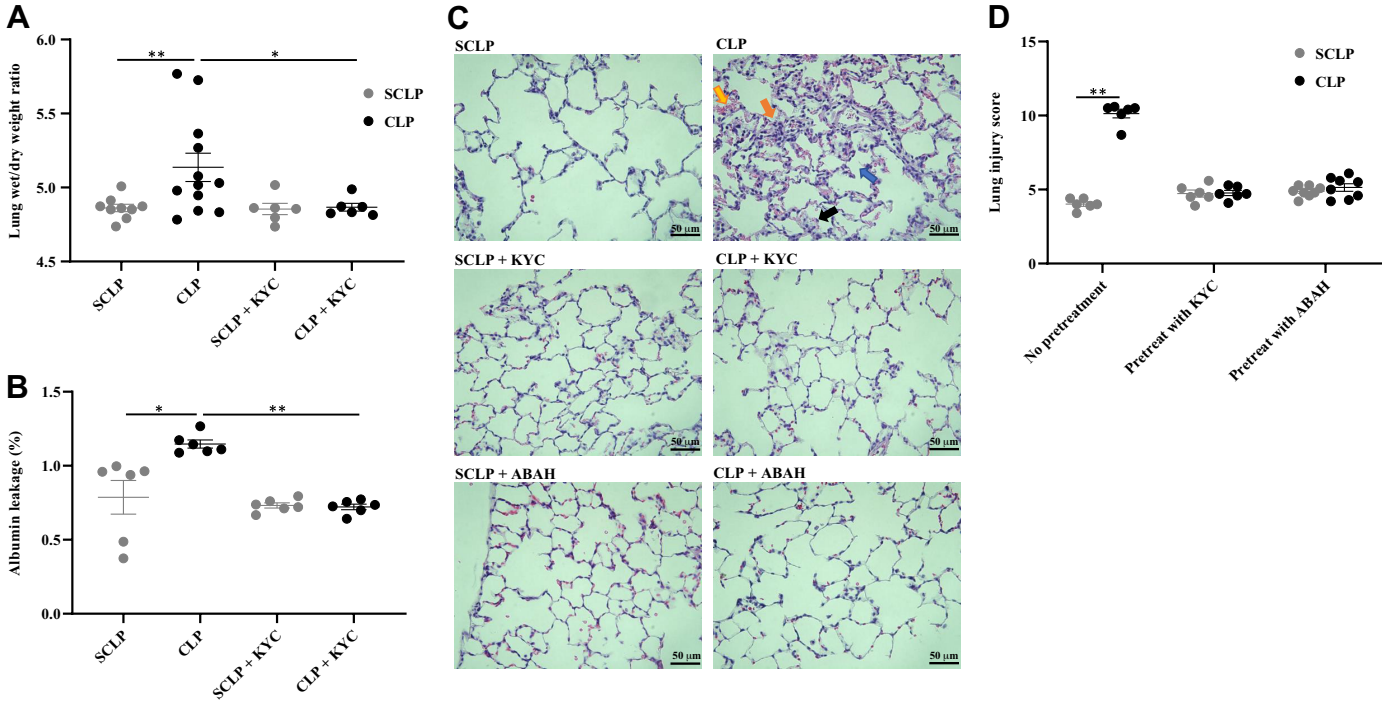

Fig. 2. Acute lung injury induced by cecal ligation and puncture (CLP) is reduced by pretreatment with myeloperoxidase (MPO) inhibitors. *A*: assessment of CLP-induced sepsis ($n = 9$) on the development of pulmonary edema, indicated by significantly increased lung wet/dry weight ratio vs. sham-operated cecal ligation and puncture (SCLP) group ($n = 12$), an effect that was prevented by pretreatment with *N*-acetyl lysyltyrosylcysteine amide (KYC). *B*: comparisons of mesenteric venular albumin leakage between SCLP and CLP groups. CLP was associated with a significant increase of albumin leakage that was abolished by KYC pretreatment. *C*: CLP-induced lung injury group showed typical findings (×400 magnification) of acute lung injury (ALI), characterized by alveolar congestion (blue arrow), intra-alveolar hemorrhage (yellow arrow), neutrophils infiltrating in the airspace and vessel wall (orange arrow), and increased thickness of alveolar wall/hyaline membrane formation (black arrow). These histologic changes induced by CLP were markedly decreased in rats pretreated with the MPO inhibitors [KYC and 4-aminobenzoic acid hydrazide (ABAH), *middle* and *bottom*]. *D*: summary of the ALI scores in different groups as indicated ($n = 8$ rats in the groups with ABAH pretreatment). Data are expressed as means ± SE. Unless otherwise stated, $n = 6$ rats. *$P < 0.05$ and **$P < 0.01$. Data in *A* and *B* were analyzed by Brown-Forsythe and Welch ANOVA tests, while the Kruskal-Wallis test was performed for lung injury scores analysis in *D*.

abrogated by pretreatment with KYC or ABAH. Injury was quantified using an injury scoring system, with results presented in Fig. 2*D*: SCLP group (4.02 ± 0.15; $n = 6$) versus CLP group (10.13 ± 0.29; $n = 6$), $P < 0.01$. The increased injury score induced by CLP was largely prevented by pretreatment with either MPO inhibitor: 4.73 ± 0.24 for SCLP + KYC, $n = 6$; 4.77 ± 0.18 for CLP + KYC, $n = 6$; 4.88 ± 0.13 for SCLP + ABAH, $n = 8$; and 5.14 ± 0.25 for CLP + ABAH, $n = 8$.

Leukocyte- and platelet-endothelium interactions. In the next series of experiments, we examined the hypothesis that MPO inhibition may prevent leukocyte rolling and adhesion induced by CLP. We first showed that these leukocyte-endothelial interactions were increased by CLP, confirming our previous observations (91). Pretreatment with either KYC or ABAH completely prevented CLP-induced leukocyte rolling (Fig. 3*A*) and adhesion (Fig. 3*B*). Taking together, these data indicate that the ability of CLP-induced sepsis to increase leukocyte rolling and adhesion is dependent on MPO. Furthermore, our results with regard to the effects of KYC and ABAH on free 2-ClFA in plasma and small intestine suggest that these chlorinated lipid species may contribute to these enhanced leukocyte-endothelial interactions, as we previously proposed (91).

Our current understanding of the pathogenesis of sepsis implicates that in addition to the effect of inflammatory cells described above, a prothrombogenic phenotype can also arise, with platelet accumulation in blood vessels (8). In light of this, we next considered the possibility that MPO promotes platelet-endothelial interactions in CLP-induced sepsis and whether pretreatment with the MPO inhibitors, KYC and ABAH, would prevent CLP-induced platelet rolling and firm adhesion along postcapillary venules. As shown in Fig. 3*C*, a large increase in the numbers of rolling platelets upon cecal ligation and puncture surgery can be readily discerned, effects that were abolished by KYC. To our surprise, pretreatment with the irreversible MPO inhibitor ABAH did not abrogate the effects of CLP to increase platelet rolling. However, the effects of pretreatment with either MPO inhibitor on platelet adhesion was more clear-cut, with both agents preventing CLP-induced platelet adhesion (Fig. 3*D*).

Mast cell activation in mesentery and PAI-1 in plasma. Because of the fact that mast cell activation can occur in sepsis and/or following provocation with 2-ClFA, which subsequently promotes leukocyte rolling and adhesion (53, 91), we next sought to determine whether this response to CLP could be abrogated by pretreatment with the MPO inhibitors. Figure 4*A* shows a summary of these experiments. CLP elicits mast cell activation by approximately fivefold compared with SCLP group. This response to CLP was abolished by pretreatment with KYC and ABAH.

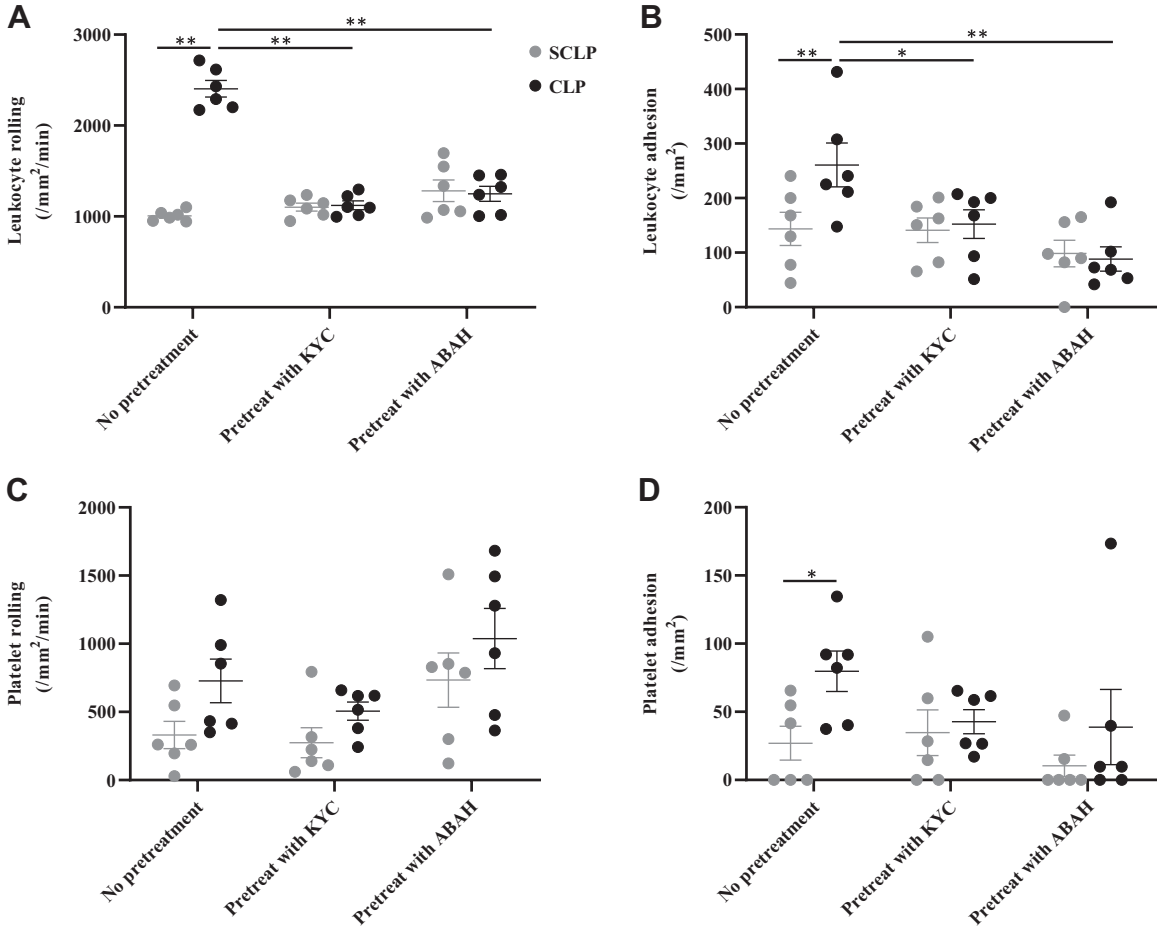

Fig. 3. Differential effectiveness of myeloperoxidase (MPO) inhibition with *N*-acetyl lysyltyrosylcysteine amide (KYC) vs. 4-aminobenzoic acid hydrazide (ABAH) on cecal ligation and puncture (CLP)-induced leukocyte-/platelet-endothelial interactions. *A*: compared with sham-operated cecal ligation and puncture (SCLP), CLP produced a marked increase in the number of rolling leukocytes. KYC pretreatment abolished this CLP-induced increase and was without effect in SCLP. Similar results were obtained in rats treated with the irreversible MPO inhibitor ABAH. *B*: CLP was associated with a significant increase in the numbers of firmly adherent leukocytes on postcapillary venules in CLP compared with the SCLP group. Both MPO inhibitors, KYC and ABAH, prevented this response in CLP animals. *C*: there was an increase (not statistically significant) in platelet rolling in CLP compared with SCLP; pretreatment with KYC, but not ABAH, abolished CLP-induced platelet rolling. *D*: CLP induced an increase in the number of firmly adherent platelets, a proadhesive response that was prevented by pretreatment with a reversible MPO inhibitor KYC or irreversible inhibitor ABAH. Data are expressed as means ± SE; $n = 6$ rats. $^*P < 0.05$ and $^{**}P < 0.01$. As data in *C* and *D* do not fit a normal distribution, nonparametric Brown-Forsythe and Welch ANOVA tests were used to analyze these data.

In light of our data implicating a role for MPO in mast cell activation in septic animals and the results of recent studies indicating that exosomes derived from activated mast cells can induce endothelial cells to secrete plasminogen activator inhibitor-1 (PAI-1), we postulated that CLP would induce an increase in tissue levels of this antifibrinolytic and proinflammatory protein (1). We next addressed the hypothesis that CLP may induce an increase in plasma levels of PAI-1. Indeed, data presented in Fig. 4*B* show that PAI-1 concentration in plasma of CLP rats was increased relative to SCLP ($P < 0.01$). Pretreatment with ABAH but not KYC prevented CLP-induced increases in plasma PAI-1.

Effects of MPO inhibition on proinflammatory cytokines (IL-1α, TNF-α, and GM-CSF) and anti-inflammatory cytokines (IL-5 and IL-10) levels. Considering that inhibition of MPO proved to be very effective in reducing acute lung injury, leukocyte and platelet adhesive interactions with endothelial cells, and mast cell activation induced by CLP, we hypothesized that MPO inhibition may limit the expression of various cytokines and chemokines that have been implicated in producing the inflammatory responses. The fact that MPO inhibition also prevented the formation of 2-ClFA and PAI-1 after CLP, proinflammatory mediators that by themselves produce a similar pattern of inflammatory responses as sepsis, supports this postulate. The data presented in Fig. 5*A* show MPO inhibition decreased CLP-induced IL-1α in mesentery. A similar pattern of response was noted with regard to CLP-induced increases in another important cytokine, TNF-α, which has been implicated as being a primary inflammatory mediator in endotoxin shock (70). Figure 5*B* summarizes the changes in TNF-α levels induced by CLP and their response to concomitant MPO inhibition. As noted with IL-1α, TNF-α levels were increased 6 h after CLP compared with SCLP, and this increase was abolished by treatment with either MPO inhibitor.

We next evaluated the effect of CLP and MPO inhibition on GM-CSF expression, a mediator that has been shown to provoke the accumulation of neutrophils in the airways exposed to TNF-α (46). Similar to the results for IL-1α and TNF-α, GM-CSF levels increased dramatically after CLP relative to those measured in SCLP (Fig. 5*C*). This CLP-induced increase

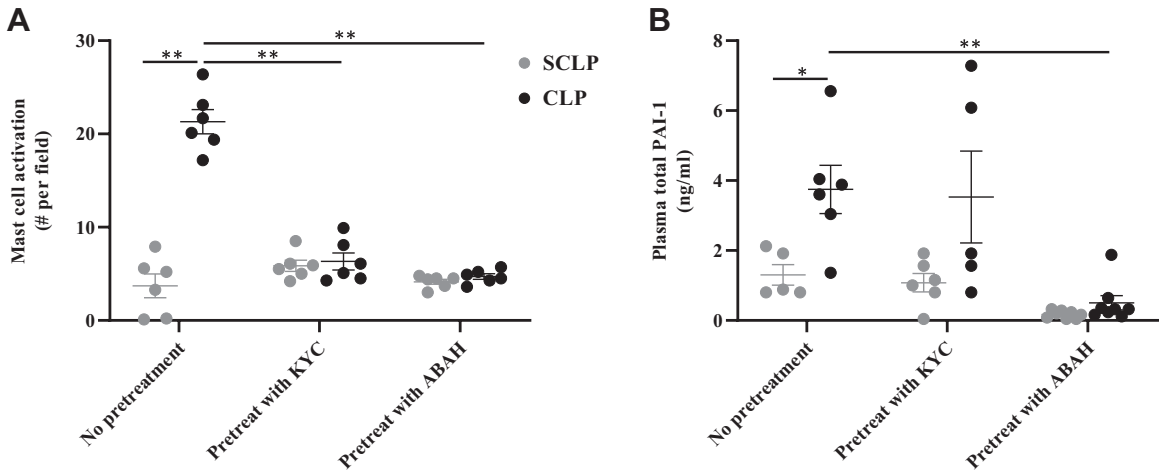

Fig. 4. Effects of *N*-acetyl lysyltyrosylcysteine amide (KYC) and 4-aminobenzoic acid hydrazide (ABAH) on cecal ligation and puncture (CLP)-induced mast cell activation and plasma total PAI-1. *A*: CLP induced a large increase in the numbers of activated mast cells relative to sham-operated cecal ligation and puncture (SCLP). These effects were abrogated by pretreatment with KYC or ABAH, respectively. *B*: animals were treated with KYC or ABAH before the SCLP or CLP to determine the effects of MPO inhibition on the increase in plasma plasminogen activator inhibitor-1 (PAI-1) levels induced by CLP. CLP increased plasma total PAI-1 relative to SCLP ($n = 5$ rats). The CLP-induced increase in plasma PAI-1 was abolished by pretreatment with ABAH (CLP + ABAH; $n = 8$ rats), but not KYC (CLP + KYC; $n = 5$). By contrast, in SCLP groups, ABAH, indeed, restricted total PAI-1 circulating in plasma (SCLP + ABAH; $n = 8$ rats), while less effect was noted in rats treated with KYC. Data are expressed as means ± SE; $n = 6$ rats, except where otherwise noted. $^*P < 0.05$ and $^{**}P < 0.01$. Brown-Forsythe and Welch tests for data analysis in *B*.

was prevented by KYC and ABAH pretreatment, indicating a role for MPO in the elaboration of this proinflammatory mediator. Thus, our data indicate that MPO is essential for the increases in CLP-induced increases in the proinflammatory cytokines IL-1α, TNF-α, and GM-CSF in the mesentery of septic animals.

While the data described above illustrate an important role for MPO in CLP-induced changes in proinflammatory cytokines, we also determined whether this enzyme influenced changes in protective anti-inflammatory cytokines, IL-5 and IL-10, after induction of CLP. In sepsis survivors, there is a trend toward elevated levels of IL-5 compared with nonsurvivors and has been reported to promote a protective innate immune response during sepsis in an eosinophil-independent manner (9, 49). Here, we report in Fig. 5*D* that CLP promotes an increase in plasma IL-5 levels compared with the SCLP surgery group. This effect was also abolished by treatment with the MPO inhibitors before induction of CLP. No differences were noted in mesenteric samples (data not shown).

Treatment with recombinant anti-inflammatory human IL-10, has been shown to regulate the progression of sepsis and control the onset of irreversible shock and mortality in CLP-induced sepsis (4, 30, 47, 74, 81). In light of these observations, we quantified the effects of CLP on levels of this anti-inflammatory cytokine in the absence and presence of MPO inhibition. As shown in Fig. 5, *E* and *F*, CLP-induced sepsis was associated with a 2-fold increase in plasma levels of IL-10 and a 1.5-fold increase mesentery levels of IL-10 compared with SCLP, effects that were abolished by pretreatment with KYC and ABAH, respectively.

Chemokine (MIP-1α, MCP-1, RANTES, LIX, IP-10, and GRO/KC) and growth factor (VEGF) levels. CLP induced increases in the expression of the proinflammatory chemokines macrophage inflammatory proteins-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1) in both plasma and mesentery (Fig. 6, *A–D*), C-C motif chemokine ligand 5 (RANTES), C-X-C motif ligand 5 (LIX), IFN-γ-inducible protein 10 (IP-10) (Fig. 7, *A–C*). As noted for proinflammatory cytokines, MPO inhibition prevented these increases induced by CLP. These results indicate that sepsis-induced chemokine expression is dependent on MPO.

In addition to increased cytokine and chemokine expression in sepsis, the results of several studies indicate that sepsis is associated with a time-dependent increase in circulating levels of vascular endothelial growth factor (VEGF) (90). Thus, we wished to determine whether expression of this growth factor might also depend on MPO in septic rats. As shown in Fig. 7*D*, CLP increased plasma VEGF to 141.48 pg/ml (versus 95.09 pg/ml for SCLP), a result consistent with earlier reports (90). This effect was attenuated by treatment with KYC or ABAH, indicating that MPO plays an important role in upregulating the expression of VEGF in septic animals.

Contribution of PAI-1 and mast cells in CLP-induced sepsis and 2-ClFALD/PAI-1 axis to sepsis. We showed that CLP was associated with increases in free 2-ClFA and PAI-1 levels, as well as mast cell activation, effects that were attenuated by MPO inhibition. To more directly evaluate whether CLP-induced inflammatory responses were dependent on PAI-1 and mast cell activation, PAI-1 inhibitor, PAI-039, and mast cell stabilizer, cromolyn sodium, were applied before SCLP and CLP surgery, as described above. The results depicted in Fig. 8, *A* and *B* show that both inhibitors attenuated leukocyte rolling and mast cell activation in sepsis, suggesting that both PAI-1 and activated mast cells act to promote inflammatory responses in CLP-induced sepsis. There is limited correlative evidence suggesting that chlorinated lipids may serve as a downstream mediator of MPO-instigated inflammation in CLP. However, since scavengers of chlorinated lipids are not currently available and cellular receptors for these lipids have yet to be identified, we took an alternative strategy to address this question by applying 2-ClFALD directly (91) in situ in the presence and absence of PAI-039 treatment. The data illus-

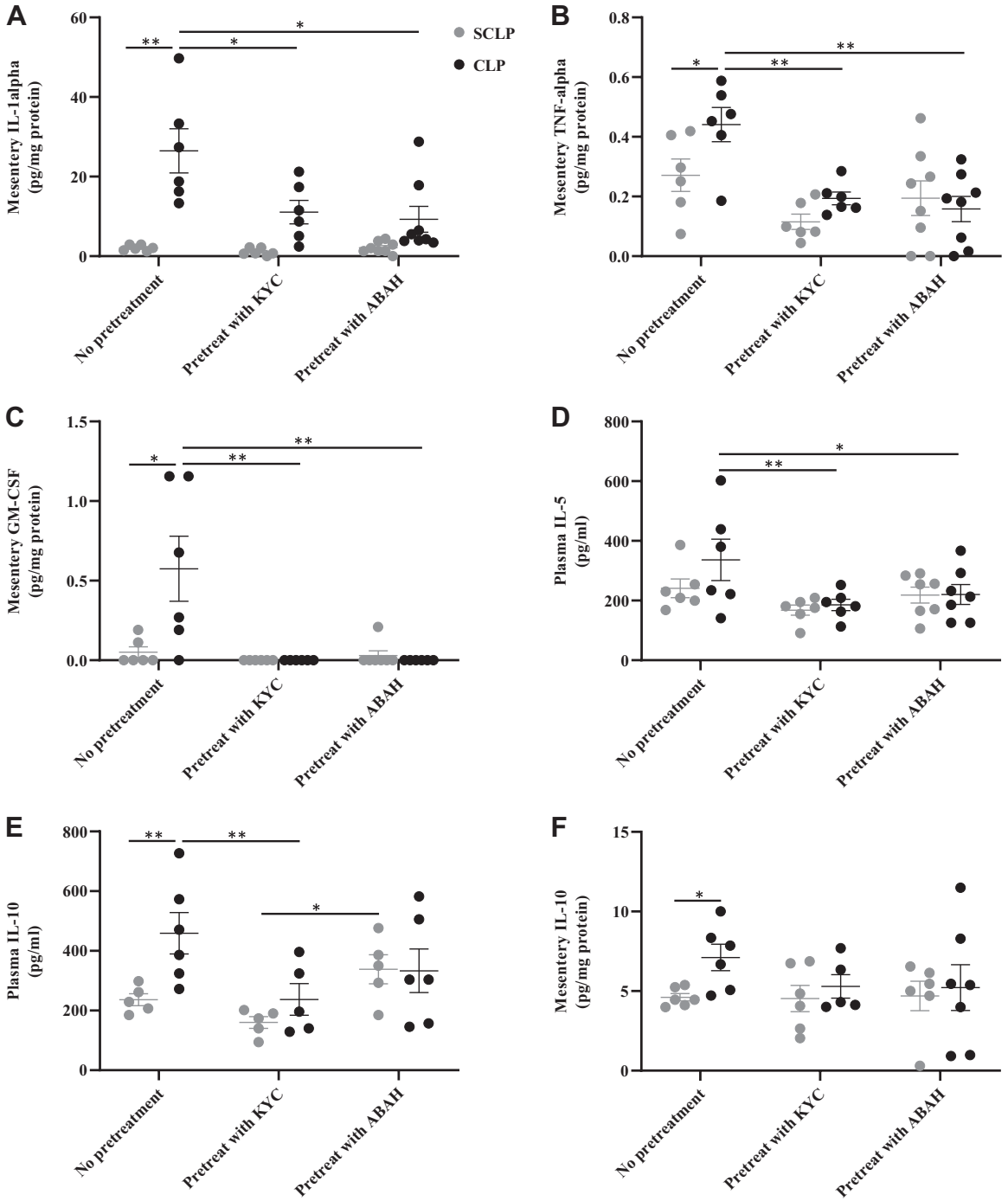

Fig. 5. Myeloperoxidase (MPO) inhibition decreases cecal ligation and puncture (CLP)-induced cytokines. *A*: CLP induced marked increases in mesenteric IL-1α, an effect that was attenuated by pretreatment with the MPO inhibitors *N*-acetyl lysyltyrosylcysteine amide (KYC) or 4-aminobenzoic acid hydrazide (ABAH). For the ABAH-pretreated groups, $n = 8$ rats. Brown-Forsythe and Welch ANOVA tests were used for analysis. *B*: CLP was associated with a significant increase in mesenteric TNF-α relative to the sham surgery. This effect was abolished by pretreatment with the MPO inhibitors KYC and ABAH. In ABAH pretreatment groups, $n = 8$ rats. *C*: CLP induced a marked increase of mesenteric granulocyte macrophage-colony stimulating factor (GM-CSF) compared with that measured in sham-operated cecal ligation and puncture (SCLP). This effect was abolished by pretreatment with KYC or ABAH, respectively (SCLP + ABAH, $n = 8$ rats). Owing to the identical numbers in KYC pretreatment groups, Brown-Forsythe and Welch ANOVA tests were inapplicable, and instead, the nonparametric Kruskal-Wallis test was applied. *D*: CLP increased plasma levels of IL-5 compared with that measured in the SCLP group. Pretreatment with KYC prevented this CLP-induced increase in IL-5 levels. A similar response was noted in rats pretreated with ABAH before CLP and SCLP. For the groups pretreated with ABAH, $n = 7$ rats. *E* and *F*: IL-10 levels increased in plasma and mesentery obtained from rats subjected to CLP vs. SCLP alone. This CLP-induced increase was abolished by pretreatment with KYC or ABAH. In analysis of IL-10 in plasma, $n = 5$ rats for SCLP, SCLP + KYC, SCLP + ABAH, and CLP + KYC group, respectively; regarding mesentery analysis (Brown-Forsythe and Welch ANOVA tests), $n = 5$ rats in CLP + KYC and $n = 7$ rats in CLP + ABAH group. Data are expressed as means ± SE; $n = 6$ rats, except otherwise specified herein. $^*P < 0.05$ and $^{**}P < 0.01$.

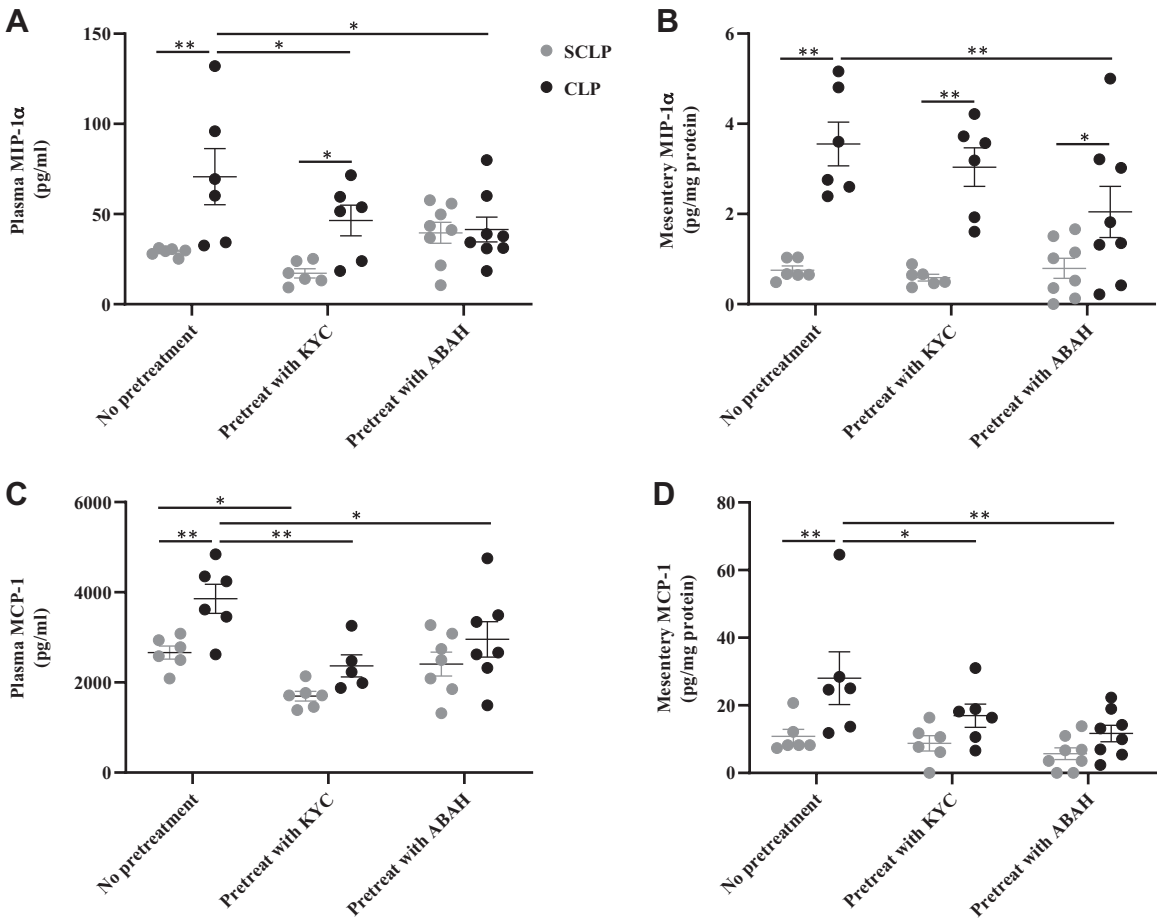

Fig. 6. *N*-acetyl lysyltyrosylcysteine amide (KYC) and 4-aminobenzoic acid hydrazide (ABAH) pretreatment attenuate increases in MIP-1α and MCP-1 induced by cecal ligation and puncture (CLP). *A*: after the sham and CLP surgery, plasma MIP-1α in sham-operated cecal ligation and puncture (SCLP) and CLP groups averaged 29.01 ± 0.89, and 70.74 ± 15.59, respectively. Following pretreatment with KYC in SCLP and CLP mice, average values were 17.16 ± 2.56 and 46.48 ± 8.51, respectively. Similarly, pretreatment with ABAH reduced macrophage inflammatory proteins-1α (MIP-1α) concentrations in plasma from both groups. For the groups pretreated with ABAH, $n = 8$ rats. *B*: a similar pattern of response was demonstrated in mesenteric tissue. Mesenteric MIP-1α levels were increased in samples obtained after CLP compared with sham surgery. ABAH was effective in reducing the increment in MIP-1α levels in mesenteric samples, while KYC was less effective in this regard. In ABAH pretreatment groups, $n = 8$ rats. *C*: comparison of the plasma monocyte chemoattractant protein-1 (MCP-1) levels for the same six groups indicated that significant increase in CLP vs. SCLP disappears with KYC or ABAH pretreatment (CLP + KYC, $n = 5$ rats; for the ABAH pretreated groups, $n = 7$ rats). *D*: similar comparisons in mesenteric MCP-1 ($n = 8$ rats in ABAH pretreatment groups; Brown-Forsythe and Welch ANOVA tests). Data are expressed as means ± SE, except for the indicated groups, $n = 6$ rats. $^*P < 0.05$ and $^{**}P < 0.01$.

trated in Fig. 8*C* show that the effect of 2-ClFALD superfusion to increase leukocyte rolling is abolished by concomitant treatment with PAI-039. The data in Fig. 8*D* illustrate that 2-ClFALD produced a substantial increase in the number of activated mast cells, an effect that was attenuated by PAI-1 inhibition. These data suggest that PAI-1 is partially responsible for mast cell activation instigated by 2-ClFALD superfusion.

In conclusion, our data support the notion that two mechanistically distinct MPO inhibitors, KYC and ABAH, effectively prevent CLP-induced *1*) increased neutrophil emigration, free 2-ClFA in plasma and small intestine, acute lung injury, leukocyte/platelet rolling and adhesion, mast cell activation, and total PAI-1 levels; 2) release of cytokines (IL-1α, TNF-α, GM-CSF, IL-5, and IL-10), chemokines (MIP-1α, MCP-1, RANTES, LIX, and IP-10), and a growth factor (VEGF) that is important in the genesis of CLP-induced lung permeability. Further exploration of PAI-1 and mast cell activation in CLP, as well as PAI-1 in exogenous 2-ClFALD superfusion, provides additional correlative support for the concept that chlorinated lipids may serve as a mechanistic link between CLP-induced MPO activity and downstream expression of these inflammatory mediators in septic animals. Taken together, these results indicate that MPO plays a key role in the production of proinflammatory responses and the development of acute lung injury in a CLP model of sepsis.

DISCUSSION

Part I. In this study, we showed that CLP caused neutrophil infiltration into the tissue space, enhanced the generation of the proinflammatory mediator 2-ClFA, produced acute lung injury, promoted the rolling and stationary adhesion of leukocytes and platelets, caused mast cell activation, and accentuated the formation of the proinflammatory mediator PAI-1. Using two structurally unrelated and mechanistically distinct MPO inhibitors, we provide some of the first evidence that this enzyme

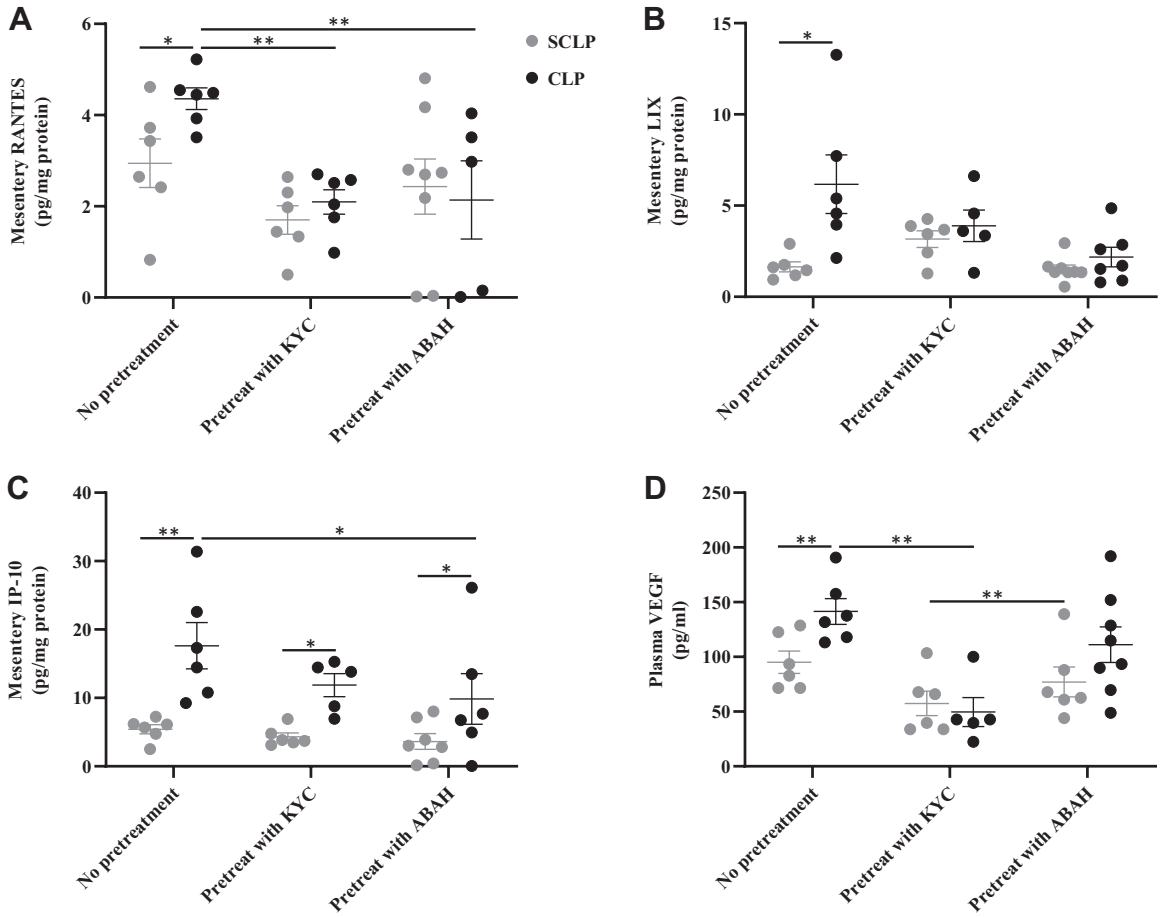

Fig. 7. Effects of myeloperoxidase (MPO) inhibitors to decrease cecal ligation and puncture (CLP)-induced expression of C-C motif chemokine ligand 5 (RANTES), C-X-C motif ligand 5 (LIX), IFN-γ-inducible protein 10 (IP-10), and vascular endothelial growth factor (VEGF). *A*: comparison of mesenteric RANTES levels between sham-operated cecal ligation and puncture (SCLP) and CLP, showing that this chemokine is increased in septic animals. In SCLP groups, the RANTES concentrations in the presence of *N*-acetyl lysyltyrosylcysteine amide (KYC) and 4-aminobenzoic acid hydrazide (ABAH) remained similar to the SCLP without pretreatment (SCLP + ABAH, $n = 8$ rats). The increased RANTES levels induced by CLP was prevented by pretreatment with KYC and ABAH (CLP + ABAH, $n = 5$ rats), indicating that MPO is required to produce the increase in mesenteric RANTES in rats subjected to CLP. *B*: CLP induced a 4-fold increase of LIX levels in mesenteries vs. that measured in rats subjected to SCLP. Pretreatment with KYC or ABAH attenuated the CLP-induced increases in mesenteric LIX levels (CLP + KYC, $n = 5$ rats; SCLP + ABAH, $n = 8$ rats; CLP + ABAH, $n = 7$ rats; Brown-Forsythe and Welch ANOVA tests). *C*: while the MPO inhibitors KYC and ABAH do not change the IP-10 concentration in response to SCLP, the increased IP-10 levels noted in mesenteries obtained from rats subjected to CLP surgery are partially decreased by KYC and ABAH (CLP + KYC, $n = 5$ rats; SCLP + ABAH, $n = 7$ rats). *D*: plasma VEGF was increased by CLP relative to that measured after SCLP. This CLP-induced increase in plasma VEGF was completely prevented by the addition of KYC (CLP + KYC, $n = 5$ rats) and partially mitigated by ABAH (CLP + ABAH, $n = 8$ rats). Plasma VEGF averaged in the groups subjected to SCLP + KYC and SCLP + ABAH were slightly decreased compared with SCLP. Data are expressed as means ± SE; $n = 6$ rats unless otherwise specified. $^*P < 0.05$, while $^{**}P < 0.01$.

instigates these inflammatory changes in this polymicrobial model of sepsis.

It is generally accepted that since circulating lipopolysaccharide released from bacteria may activate both neutrophils and monocytes, increased MPO enzyme activity in plasma could be an indicator of inflammation and the onset of sepsis (44). Against all expectations, MPO-derived 2-ClFA showed a stronger correlation with either the development of ARDS or 30-day mortality in the course of sepsis, rather than MPO levels per se (53). In addition, we presented evidence that in intact rat mesentery, exogenous chlorinated lipids, including 2-ClFALD and its metabolite 2-ClFA elicit inflammatory responses in microcirculation that strongly mimic those that characterize sepsis (91). Here, we will first discuss the direct role of chlorinated lipid species generated in sepsis based on our current understanding of MPO/HOCl/chlorinated lipid system in activated neutrophils, and then employ MPO inhibitors to interfere the production of these chlorinated lipids to establish correlative evidence that these events may be linked. In addition, our results suggest that activation of mast cells and the expression of PAI-1 may serve as downstream mediators of the inflammatory responses elicited by MPO-derived chlorinated lipids.

In this study, we used two structurally unrelated and mechanistically distinct MPO inhibitors to probe a role for this enzyme in sepsis-induced CLP, as described in the introduction. Because many known MPO inhibitors are very toxic, can be metabolized to toxins, or are easily oxidized with loss of function (27), recent work has focused on designing new MPO inhibitory reagents that exert less toxicity and resist oxidation. KYC is one of these newer-generation competitive MPO inhibitors, wherein tyrosine moieties outcompete Cl^- or NO_2^- for binding to prevent MPO from generating HOCl and $\cdot NO_2$,

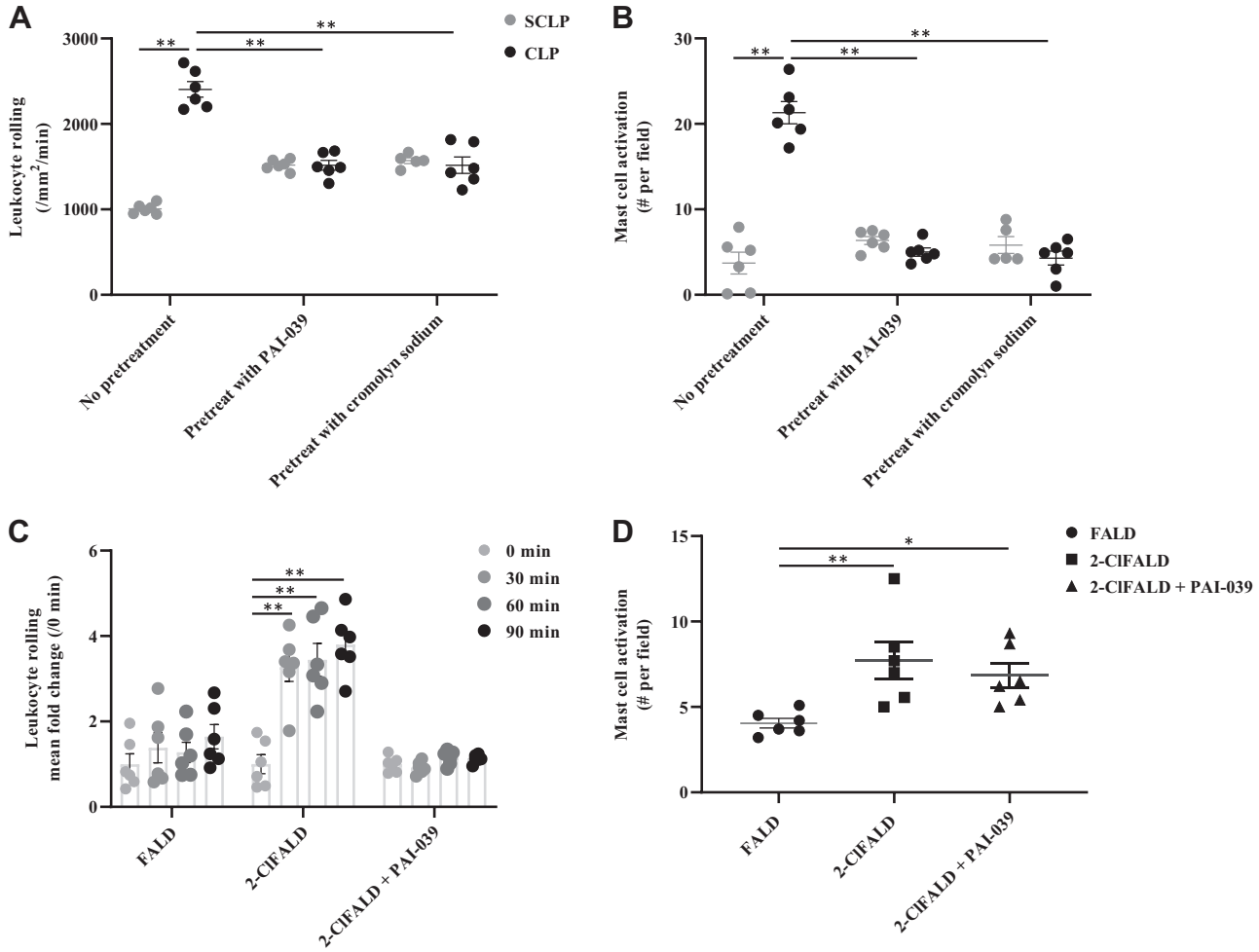

Fig. 8. Regulation of cecal ligation and puncture (CLP)-induced systemic inflammation and chlorinated lipid-elicited localized response. *A* and *B*: CLP remarkably increases the number of rolling leukocytes and mast cell activation, which were attenuated by pretreating rats with plasminogen activator inhibitor-039 (PAI-039), or the mast cell stabilizer (cromolyn sodium). In sham-operated cecal ligation and puncture (SCLP) + cromolyn sodium group, $n = 5$; Brown-Forsythe and Welch ANOVA tests were performed in *B*. *C*: effects of PAI-039 on chlorinated lipid superfusion-induced leukocyte rolling. The intact rat mesentery was challenged with exogenous fatty aldehyde (FALD) (negative control), 2-chlorofatty aldehyde (2-ClFALD) (positive control), and 2-ClFALD superfusion with PAI-039 pretreatment. Despite that the mean fold change of leukocyte rolling in FALD group rises very slightly at 30, 60, and 90 min as against to baseline (0 min), 2-ClFALD superfusion promoted much more significant leukocyte rolling at the matching time points, effects that were abolished by PAI-039 pretreatment. *D*: evaluation of mast cell activation in mesenteric interstitium after 90 min of superfusion. 2-ClHDA provoked enhanced mast cell activation when compared with the FALD group, which was attenuated by treatment with PAI-039 before the initiation of 2-ClFALD superfusion. Data are expressed as means ± SE; $n = 6$ for the rest groups. *$P < 0.05$ and **$P < 0.01$.

conferring specificity in targeting MPO as an inhibitor (92). Importantly, no toxic effects have been observed in three different disease models. Given its specificity for MPO and apparent lack of toxicity, we selected KYC as an ideal reversible MPO inhibitor candidate for use in our CLP-induced sepsis model (71, 93, 94). Being mechanistically distinct from and structurally unrelated to KYC, we also employed the irreversible inhibitor of MPO (52), ABAH, in these studies as a second approach to interrogate the role of this enzyme in sepsis-induced inflammatory responses. ABAH inhibits both the chlorination and peroxidation activities of MPO (25).

We first determined whether treatment with MPO inhibitors would prevent the infiltration of MPO-positive cells (primarily neutrophils at the time point assessed in our studies) in our CLP model. KYC and ABAH pretreatment markedly inhibited MPO expression in the small intestine 6 h after induction of sepsis. These observations (Fig. 1, *A* and *B*) suggest that MPO participates directly in the processes mediating neutrophil transmigration into the tissues or is required for the formation of chemotactic agents and/or elaboration of adhesion molecules that participate in leukocyte rolling and adhesion, all of which are required to promote leukocyte infiltration into the tissues.

We have previously suggested that sepsis may drive the generation of chlorinated lipids derived from MPO-dependent HOCl production, which, in turn, may act as inflammatory mediators to elicit leukocyte-endothelial interactions (53, 91). In the present study, we showed that CLP was associated with a modest increase in plasma levels of free 2-ClFA (18:0 Cl), as shown in Fig. 1*C*. While the increase in plasma is relatively small, it is likely that 2-ClFA levels were higher at sites of production and are diluted in the systemic blood. It is also possible that our modified CLP sepsis model, which maintained a patent mesenteric microcirculation for intravital mi-

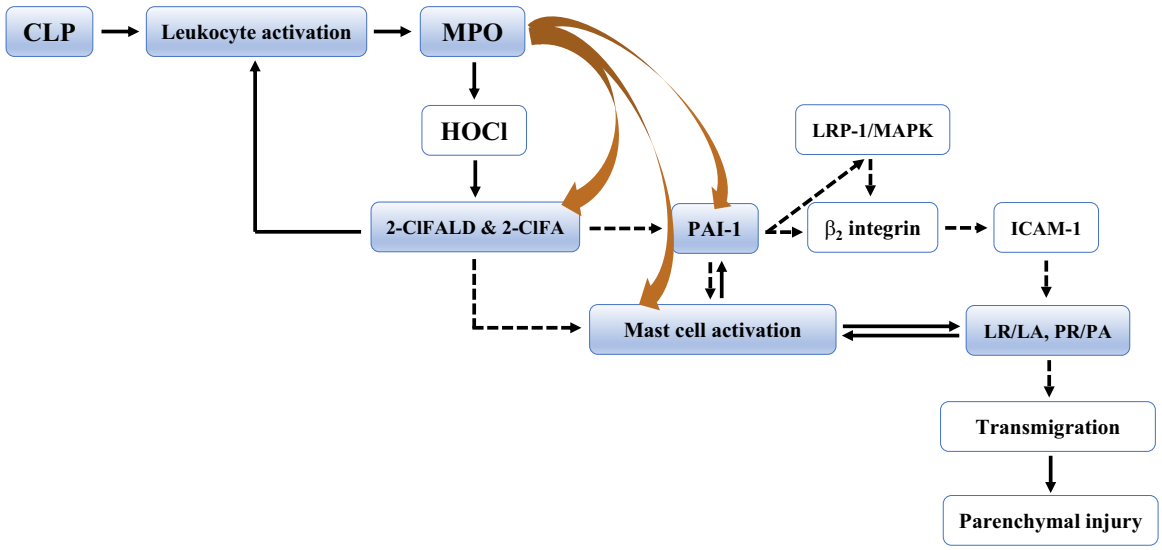

Fig. 9. Schematic view explaining the effects of myeloperoxidase (MPO) inhibitors on downstream effectors. Our results indicate that MPO inhibition prevents cecal ligation and puncture (CLP)-induced production of 2-chlorofatty acid (2-ClFA), plasminogen activator inhibitor-1 (PAI-1) expression, mast cell activation, leukocyte and platelet adhesive interactions [leukocyte rolling and adhesion (LR/LA) and platelet rolling and adhesion (PR/PA)], neutrophil infiltration, and acute pulmonary injury. Dashed arrows represent the focus of further investigation in future studies. See text for further explanation. ICAM, intercellular adhesion molecule 1; LRP-1, low-density lipoprotein receptor-related protein 1; MAPK, mitogen-activated protein kinase.

croscopy observation, may not have generated the same level of inflammatory effects compared with standard CLP sepsis models. Given the modest increase of 2-ClFA in the small intestine in our subsequent investigation, but their severalfold greater increase in a cecal slurry model of sepsis from our collaborator's laboratory (61), supports the latter proposal. Nevertheless, the significant reduction of free 2-ClFA (18:0 Cl) by KYC and ABAH, provides support for the notion that MPO activity plays an important role in fueling the formation of 2-ClFA (18:0 Cl) in sepsis.

Bioactive chlorinated lipids have been implicated as potential mediators of ARDS, a devastating complication of sepsis that results in extremely high mortality (3, 21, 42, 57). Although our understanding of the pathologic events contributing to the development of ARDS occurring in septic subjects continues to evolve, it is clear that pulmonary barrier dysfunction, secondary to neutrophil recruitment, and activation and excessive free radical production (26) are the major contributing processes. However, the role of the MPO/HOCl/chlorinated lipid axis in producing pulmonary barrier dysfunction has not been, heretofore, evaluated. In our studies, we first verified the development of pulmonary edema and permeability change characterized by increased lung wet/dry weight ratio (Fig. 2*A*) and mesenteric albumin leakage (Fig. 2*B*) in CLP-induced sepsis. Since these changes were prevented by pretreatment with the MPO inhibitor KYC before CLP, it appears that sepsis-induced MPO activation is an important mediator of pulmonary edema and systemic permeability changes. To more thoroughly address the role of MPO in lung-induced injury, we evaluated the effects of MPO inhibition on CLP-induced alveolar congestion, intra-alveolar hemorrhage, pulmonary neutrophil infiltration, and increased thickness of the alveolar wall. KYC or ABAH treatment before CLP largely abolished these pulmonary changes (Fig. 2, *C* and *D*). Taken together with our observations that MPO inhibition prevents the increase in plasma 2-ClFA levels, these data support an important role for MPO, and perhaps 2-ClFA, in the development of pulmonary dysfunction induced by CLP.

Previous work from our group indicated that 2-ClFA leads to mobilization of Weibel-Palade bodies and the release of ANG II, surface expression of P selectin and E selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (53, 91). These effects were associated with an increased adhesive interaction of neutrophils and platelets with endothelial cells. In the current study, CLP-induced increases in the numbers of rolling and adherent leukocytes and platelets that mimicked these responses to 2-ClFA. Importantly, administration of KYC and ABAH before induction of CLP prevented these sepsis-induced adhesive interactions, except that ABAH failed to prevent platelet rolling (Fig. 3, *A–D*). It is possible that higher ABAH doses would be required to prevent platelet rolling, but we cannot address this question owing to protective effects from the larger amounts of DMSO diluent required to solubilize this inhibitor at higher doses. We also speculate that MPO-dependent firm platelet adhesion may partially account for development of disseminated intravascular coagulation (DIC) in sepsis.

It is still controversial regarding whether the transmigrated leukocytes secrete mediators to initiate mast cell activation in sepsis or whether mast cell activation occurs first, with subsequent release of mast cell mediators acting to promote neutrophil diapedesis (19, 36–38). In earlier work (91), we showed that chlorinated lipids elicit mast cell activation. On the basis of this observation, we tested the hypothesis that MPO inhibition, and its effect to prevent the formation of HOCl required to produce chlorinated lipids, would limit CLP-induced mast cell activation involved in sepsis. Our data (Fig. 4*A*) support this hypothesis in that both MPO inhibitors (KYC or ABAH) prevented mast cell activation in our septic model. Taken together, our data are consistent with the notion that 2-ClFA generated from MPO/HOCl system in activated neutrophils initiates inflammatory cascades in sepsis by promoting mast

cell activation. Figure 9 presents a summary hypothesis diagram that integrates these responses to CLP.

Owing to its antifibrinolytic actions, PAI-1 may play a role in disseminated intravascular coagulation in sepsis (51). In addition, recent work has shown that ischemia/reperfusion causes immobilization of PAI-1 secondary to binding to cell surface glycosaminoglycans on neutrophils. This binding provokes affinity changes in β_2 integrin on rolling neutrophils to facilitate the transition to stationary neutrophil adherence and subsequent transmigration (45, 75). In addition, PAI-1 participates in the regulation of vascular permeability (55, 89), either directly or by its effect to recruit neutrophils (16, 65). MPO inhibition with ABAH before CLP abolished increases in plasma PAI-1 levels that normally occurs in sepsis (Fig. 4*B*). However, KYC was ineffective in this regard, a result that is difficult to explain given the fact that KYC abolished all other proinflammatory responses to CLP, unless higher KYC doses are required to block this particular effect of MPO, as discussed above.

Although our data in part I clearly implicate a role for MPO in the proinflammatory responses to CLP, it is difficult to address the direct involvement of 2-ClFA derived from MPO-generated HOCl. This relates to the fact that agents that specifically scavenge chlorinated lipids have not been identified. Thus, we have relied on a correlative analysis to support this link. However, this interpretation should be viewed with caution until the link between MPO-generated 2-ClFA as an instigator of downstream responses can be directly tested by use of such scavenging agents.

Although there was some inconsistency with regard to the effectiveness of both inhibitors in abolishing the effects of CLP-induced, MPO-dependent activation of downstream proinflammatory and prothrombogenic effects of sepsis, our data are consistent with the scheme shown in Fig. 9, which may be used as a guide for future studies that require additional work to substantiate or refute. Our data in part I strongly support the concept that CLP-induced MPO activity is required for increased neutrophil emigration, plasma and small intestine levels of 2-ClFA, acute lung injury, leukocyte and platelet rolling and adhesion, and mast cell activation in sepsis. The potential role of mast cells and PAI-1 in these responses to CLP will be more directly addressed by using pharmacologic inhibitor approaches in future studies.

Part II. Determining cytokine and chemokine expression patterns in sepsis should provide critical insights regarding the complex interactions of inflammatory mediators in this devastating disorder. In the present study, we characterized circulating and tissue cytokine profiles following SCLP and CLP by multiplex assay analysis. To perform this expression profile, we first need to ask: what time following surgery is a good starting point for this analysis? With reference to our findings, significant neutrophil activation and the pertinent tissue MPO accumulation (Fig. 1, *A* and *B*), greater plasma and small intestine 2-ClFA (18:0 Cl) (Fig. 1*C*), advanced acute lung injury (Fig. 2, *A* and *B*), enhanced leukocyte and platelet adhesive interactions (Fig. 3), *A–D*, intensified mast cell activation and PAI-1 expression (Fig. 4, *A* and *B*) were noted at 6 h after CLP. Since all these inflammatory responses are likely to be modulated by cytokine and chemokine expression, we felt that assessing cytokine and chemokine expression 6 h after CLP surgery would be an important first time point to assess. As expected, we found increased expression of a number of cytokines (IL-1α, TNF-α, GM-CSF, IL-5, and IL-10), chemokines (MIP-1α, MCP-1, RANTES, LIX, and IP-10), and the growth factor (VEGF) in rats subjected to CLP. It is important to emphasize that these changes pertain to our specific CLP model as the responses to CLP can vary depending on the specific procedural details [e.g., portion of cecum affected by ligation, degree (partial vs. complete) of the ligation, size of needle used to perforate the cecum and where in the cecum the perforation occurs, and number of punctures] (68).

Because distinct cytokine profiles are associated with sepsis severity, organ failure, and mortality (9), current literature offers no consensus opinion, as there are multiple studies both in support and against the utility of cytokines as prognostic biomarkers (83). Regardless of relative lack of sensitivity and specificity for single cytokines as markers of sepsis severity, overabundant generation of early proinflammatory mediators, notably IL-1 and TNF-α, indeed contribute to the unfavorable consequences following sepsis (7, 82) and have the ability not only to induce expression of other chemokines and inflammatory mediators, but also induce endothelial cell dysfunction and prothrombotic events (15, 22, 28, 48, 59, 80). Moreover, Ertel et al. (20) induced sepsis by CLP and observed a persistent elevation of plasma TNF-α and a steadily increasing IL-1 plasma concentrations compared with the sham group.

The classical view that neutrophils function as simple professional killers of invading pathogens (56) has been expanded by the acknowledgment that appropriately activated neutrophils constitute a substantial source of a variety of secreted cytokines, supporting a direct contribution of these cells in the regulatory framework of the adaptive immune response (10, 39, 72). It is now recognized that increased release of neutrophil-derived cytokines contribute to the inflammatory responses in sepsis by promoting overexuberant activation/amplification of host defenses, resulting in damage to host tissue (29). Given the facts that 2-ClFA and PAI-1 can reproduce similar proinflammatory changes as both sepsis and many cytokines and chemokines, we postulated that CLP would cause increases in cytokine and chemokine expression that would be attenuated by treatment with MPO inhibitors. If this postulate would prove to be supported by our experiments, it would suggest that MPO plays a critical role in upregulating their expression versus the commonly held view that invading microorganisms directly induce cytokine expression.

Interestingly, Endo et al. (18) provided the evidence that MPO negatively regulates the expression of proinflammatory cytokines and chemokines, such as MIP-1α, MIP-1β, IL-1α, IL-1β, and TNF-α, by zymosan-activated mouse neutrophils. This result suggests that MPO differentially regulates cytokine/chemokine expression, depending on the stimulus for neutrophil activation and subsequent stimulation of NADPH oxidase and MPO. Whatever the explanation, our results allow us to propose that MPO activation secondary to CLP is directly involved in the upregulated expression of a number of cytokines, chemokines, and a growth factor, which, in turn, augment proadhesive responses between leukocytes, platelets, and the endothelium, and neutrophil accumulation in the tissues that may also involve MPO-dependent formation of chlorinated lipids, mast cell activation, and PAI-1 expression (Fig. 10).

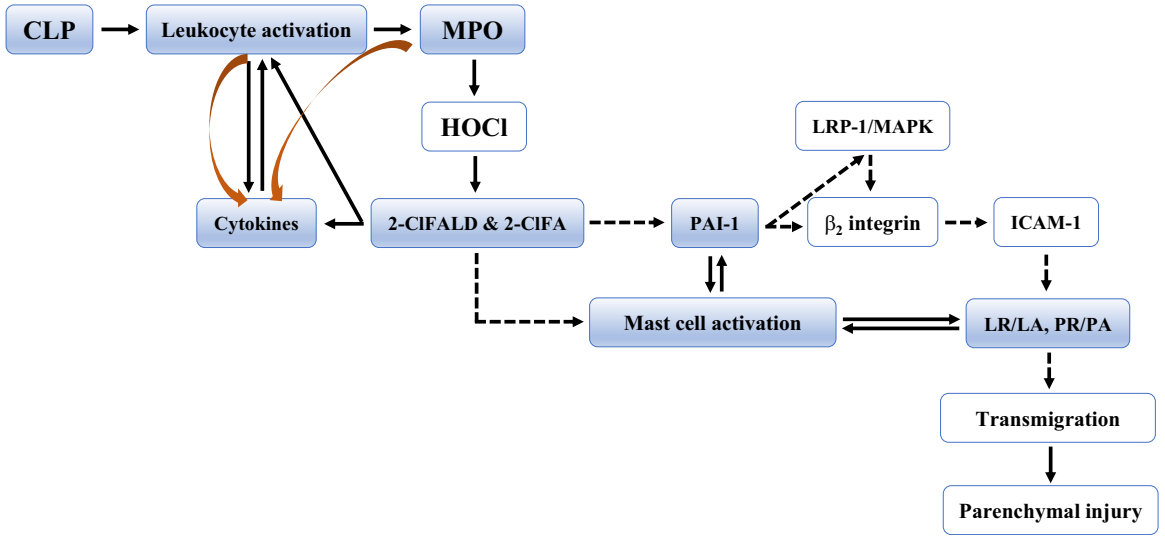

Fig. 10. Regulation of cytokines by myeloperoxidase (MPO)-derived free 2-chlorofatty acid (2-ClFA). Scheme introduces cecal ligation and puncture (CLP), cytokine production, and the ensuing responses. Brown arrows denote that inflammatory stimulus, ligand binding via Toll-like receptors (TLRs), and damage-associated molecular patterns (DAMP) receptors can promote the cytokine production on one hand. On the other hand, in light of similar change patterns of 2-ClFA and cytokine production, we propose that MPO-derived free 2-ClFA may also participate in CLP-induced cytokine/chemokine generation. ICAM-1, intercellular adhesion molecule 1; LR/LA, leukocyte rolling and adhesion; LRP-1, low-density lipoprotein receptor-related protein 1; MAPK, mitogen-activated protein kinase; PR/PA, platelet rolling and adhesion.

In the current study, we demonstrate that in sepsis, increased cytokine secretion can be modulated by MPO inhibitors such as KYC and ABAH. When we analyzed the corresponding cytokines for each inhibitor in the four experimental categories, we found that increases of most of the cytokines assessed can be prevented by the pretreatment with KYC or ABAH. Specifically, the three proinflammatory cytokines exhibiting the largest increases after CLP, IL-1α, TNF-α, and GM-CSF, were not elevated in groups treated with the MPO inhibitors (Fig. 1, *A*–*C*). For the anti-inflammatory cytokines IL-5 and IL-10, inhibition of MPO with KYC and ABAH was also effective in preventing their increase after CLP (Fig. 1, *D*–*F*). These results, thus, led us to the conclusion that upregulation of pro- (IL-1α, TNF-α, and GM-CSF) and anti-inflammatory cytokines (IL-5 and IL-10) by CLP requires functional MPO, and suggested the possibility that MPO-dependent expression of IL-5 and IL-10 may serve as a brake to reduce the proinflammatory effects of other cytokines and chemokines in sepsis.

It is well known that noxious stimuli lead to rapid production of proinflammatory cytokines and chemokines, such as MCP-1, MIP-1, and RANTES (60), which play important roles in regulating the magnitude and sequence of leukocyte trafficking during inflammatory response (50, 69). In addition, it is well established that cytokine-induced cytokine expression can occur. For example, IL-1 and TNF-α induce the expression of MIP-1 (5, 6, 12, 40, 88). This is analogous to our suggestion that MPO-derived chlorinated lipids may provoke the release of cytokines and chemokines. Similar analysis was performed to test the changes in the plasma VEGF induced by CLP and whether the expression of this growth factor can be prevented by pretreatment with MPO inhibitors. This is an important observation because it has been suggested that sepsis-induced VEGF production may contribute to microvascular barrier disruption (90).

In this study, we present data suggesting that PAI-1 and mast cell activation participate in the genesis of proinflammatory responses to CLP via an MPO-dependent mechanism that may involve generation of chlorinated lipid as a downstream activator of these inflammatory responses. Interestingly, both PAI-1 per se and mast cell activation by chemical compounds in lieu of CLP have been shown to exert potent chemoattractant activities for neutrophils and occur in postischemic tissues (2, 79). We have extended these observations to sepsis by showing that the proinflammatory responses to CLP are prevented by treatment with a PAI-1 inhibitor (PAI-039) or mast cell stabilizer (cromolyn sodium) (Fig. 8, *A* and *B*). More profoundly, HOCl produced by neutrophil MPO has been shown to target plasmalogen in neighboring endothelial cells and extracellular lipoproteins, leading to 2-ClFALD production, and endothelial cells metabolize 2-ClFALD to 2-ClFA, which is subsequently released from these cells. These observations led to the proposal that free 2-ClFA may function as a biomarker of chlorinated lipid production in sepsis, whereas 2-ClFALD is the actual initiator of the inflammatory responses to sepsis (77, 78, 86). In view of the effect of sepsis to cause 2-ClFA generation, as shown in Fig. 8, *C* and *D*, our studies regarding the contributions of PAI-1 and mast cell activation to enhanced leukocyte-endothelial interactions induced by exogenous chlorinated lipid (2-ClFALD) provide new correlative evidence for a potential role for chlorinated lipid as a potential mediator for pathophysiological responses to sepsis.

Although we postulate that chlorinated lipid generation secondary to MPO activation may be a direct mediator of cytokine/chemokine expression, this is based on a correlative analysis that is not sufficiently rigorous to substantiate this claim. To address this issue will require development of compounds that act to specifically scavenge chlorinated lipids as they are produced. Another possible approach lies in identifying potential receptors for chlorinated lipids and using a

receptor blocker to address the question. In this regard, one possibility is that chlorinated lipids may use the scavenger receptor CD36 to initiate responses (58, 95). Finally, we believe that examination of the changes in cytokine expression patterns in response to CLP, together with the molecular insights regarding the role of the MPO/HOCl/2-ClFA in CLP provided in *Part I* may provide new insights for early detection and intervention of sepsis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

H.Y., Y.J.L., D.A.F. and R.J.K. conceived and designed research; H.Y., Y.J.L, M.F.W., R.J.R., D.Z.W. and T.J.K. performed experiments; H.Y. and Y.J.L. analyzed data; H.Y. interpreted results of experiments, prepared figures, and drafted manuscript; D.A.F and R.J. K. edited and revised manuscript; H.Y., Y.J.L., M.F.W., R.J.R., D.Z.W., T.J.K., W.L.N., D.A.F. and R.J.K. approved final version of manuscript.

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