MECHANISMS FOR CHLORINATED LIPIDS IN SEPSIS

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Hong Yu

Dr. Ronald J. Korthuis, Dissertation Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

MECHANISMS FOR CHLORINATED LIPIDS IN SEPSIS

presented by Hong Yu, a candidate for the degree of Doctor of Philosophy of Medical Pharmacology and Physiology, and hereby certify that, in their opinion, it is worthy of acceptance.

_____________________________________________________
Dr. Ronald J. Korthuis, Medical Pharmacology and Physiology

_____________________________________________________
Dr. Virginia H. Huxley, Medical Pharmacology and Physiology

_____________________________________________________
Dr. David A. Ford, Biochemistry and Molecular Biology, SLU

_____________________________________________________
Dr. Zezong Gu, Pathology and Anatomical Sciences

_____________________________________________________
Dr. Alan R. Parrish, Medical Pharmacology and Physiology
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MECHANISMS FOR CHLORINATED LIPIDS IN SEPSIS

Hong Yu

Dr. Ronald J. Korthuis, Dissertation Advisor

ABSTRACT

Sepsis induces the formation of proinflammatory chlorinated lipids, stimulates adhesive interactions between the endothelium and leukocytes/platelets, causes mast cell activation, disrupts the microvascular barrier and causes acute lung injury. Using a cecal ligation and puncture (CLP) model of polymicrobial sepsis, the aims of this study were to determine whether: 1) the proinflammatory responses to sepsis are instigated by myeloperoxidase (MPO), an enzyme that produces hypochlorous acid, which can attack membrane plasmalogens to produce chlorinated lipids, 2) MPO plays an important role in the generation of cytokines and chemokines that have been proposed as mediators of the inflammatory responses to sepsis, and 3) plasminogen-activator inhibitor-1 (PAI-1) and mast cells contribute to CLP-induced adhesive interactions between leukocytes/platelets and the endothelium. A subproject to Aim 3 sought to identify the contribution of PAI-1 and mast cells to adhesive interactions induced by chlorinated lipids in lieu of CLP, as an approach to support the concept that MPO-derived chlorinated lipids may serve as an important mediator of sepsis-induced inflammatory responses. Our results are consistent with the concept the CLP promotes MPO-dependent release of proinflammatory PAI-1, cytokines, and chemokines, which in turn activate mast cells to elicit leukocyte/platelet adhesive interactions with the endothelium, neutrophil migration into the tissues and disruption of microvascular barrier function.
CHAPTER I: INTRODUCTION

i. A brief history of sepsis

Sepsis is a potentially lethal complication of bacterial invasion that is known for its characteristic life-threatening organ dysfunction, driven by dysregulated host response to infection. Although the very first mention of sepsis as a dangerous disorder was provided by Hippocrates in 400 BC (Funk et al., 2009), it is not until the development of germ theory in the 1800s that a series of studies demonstrated that pathogens entering the body were responsible for the development of sepsis (Vincent et al., 2006). Despite the evidence-based guidelines for the appropriate management that were developed over time, delayed or failure to identify sepsis as it develops is key to subsequent decline in patient status. As such, mortality in sepsis is still unacceptably high (Keeley et al., 2017; Noah, 2014). Therefore, a search for reliable biomarkers as a means of early recognition of sepsis began to emerge (Marshall and Reinhart, 2009; Tracey et al., 1986; Tracey et al., 1987), and studies to define sepsis pathogenesis regarding the balance of pro- versus anti-inflammatory cytokines were addressed (Glauser et al., 1994; Walley et al., 1996).

ii. Leukocytes in the pathogenesis of sepsis

Leukocytes play a pivotal role in inflammation given that they form the first line of defense against infection. In opposition to local inflammation, systemic sepsis is featured by profound leukocyte activation throughout the circulation (Chishti et al., 2004; Kaufmann et al., 2006). Indeed, as regards leukocyte transcriptional responses in sepsis, RNA sequencing revealed that 916 unique mRNA transcripts differentially expressed during sepsis. Moreover, functional enrichment analysis disclosed a highly dynamic
downstream effect of the transcriptional activity during sepsis that, of the 43 cellular pathways activated during sepsis, several were closely associated with inflammation and response to infection (Skibsted et al., 2019). As a result, it is now accepted that activated neutrophils interact improperly with endothelial cells and prematurely degranulate, releasing lytic enzymes and oxygen radicals to cause endothelial dysfunction (Weiss, 1989). Endothelial barrier disruption results in enhanced fluid and protein leakage, which becomes especially problematic in the lungs, where gas exchange becomes limited. In all organs of septic patients, increased expression of adhesion molecules on circulating leukocytes and endothelial cells facilitates their interactions and results in leukocyte infiltration into the tissues, where they cause cellular damage as a by-product of their attack on bacteria (Kobold et al., 2000). Systemic leukocyte activation and disseminated leukocyte adhesion are regarded essential for septic organ dysfunction, a concept supported by studies showing that organ damage is attenuated by inhibiting leukocyte-endothelial interactions (Laschke et al., 2007; Liu and Kubes, 2003; Reutershan and Ley, 2004; Watanabe et al., 1995).

Neutrophils are the first and most abundant leukocytes to arrive at the infection focus. These inflammatory phagocytes are ideally suited to eliminate microbes due to 1) their capacity to release a large number of hydrolytic enzymes, 2) rapid production of reactive oxygen species, and 3) generation of reactive nitrogen species (Seely et al., 2003). More recently, we provided evidence that chlorinated lipids, produced secondary to activation of myeloperoxidase by activated leukocytes, may also participate in inflammatory responses to sepsis (Yu et al., 2019). Septic neutrophil recruitment into the tissues occurs almost exclusively in postcapillary venules and is mediated by a combination of
mechanical, chemical, and molecular processes (Alves-Filho et al., 2008). Distinct 
procedures are linked sequentially: First, margination along the postcapillary venular 
walls exposes these cells to proinflammatory mediators which cause the upregulation 
and/or activation of adhesive molecules on their surface, which facilitates leukocyte 
rolling. This movement of the neutrophils from the central stream to the venular wall is 
dependent on hydrodynamic forces. These marginated cells can then form weak adhesive 
interaction dependent on selectins and their corresponding ligands and roll along the 
venular surface (Kansas, 1996). These rolling cells monitor their local environment for 
the presence of activating factors that facilitate the transition to stationary adhesion, a 
much stronger adhesive interaction mediated by integrins expressed on leukocytes and 
intercellular adhesion molecule-1 (ICAM-1) on endothelial cells (Huttenlocher et al., 
1995). These firmly adherent cells can then transmigrate into the tissues, a process that 
requires a chemoattractant gradient for directional cues (Muller, 2003). Of note, the 
specificity of chemokine-induced leukocyte chemotaxis is related to differential 
extpression of chemokine receptors on specific leukocyte subsets, mechanisms that allow 
the host response to deliver specific subsets of leukocytes to localized areas of infection 
(Moser et al., 2004).

iii. MPO-derived chlorinated lipids in sepsis

Although studies (Anger, 1941) first described myeloperoxidase (MPO) as a highly 
abundant protein in neutrophils in 1940s, it is not until two decades later that MPO was 
purified (Agner, 1958). Subsequent work demonstrated that this enzyme produces 
hypochlorous acid (HOCl), a reactive species that can chlorinate biomolecules, altering 
their function. Release of MPO from neutrophils at or near the endothelial glycocalyx
leads to its accumulation along the endothelium, followed by transport across endothelial
cells (Tiruppathi et al., 2004). Subsequent MPO-induced changes include targeting
extracellular matrix proteins for oxidative modification and/or cleavage, reduced NO
bioavailability, and neutrophil recruitment and activation (Baldus et al., 2001; Baldus et
al., 2004; Eiserich et al., 2002; Klinke et al., 2011; Lau et al., 2005). Another deleterious
function of MPO is that the enzyme stimulates the shedding of syndecan-1 resulting in
glycocalyx breakdown. This occurs because MPO possesses more than 70 cationic amino
acids, allowing it to bind to and destabilize the negatively charged endothelial glycocalyx
(Manchanda et al., 2018).

Despite the fact that MPO can mediate endothelial dysfunction via the aforementioned
mechanisms, its main physiological role is contributing to MPO-halide-H2O2 system as
an antimicrobial defense that regulates phagocytosis (Klebanoff, 1968; Klebanoff, 1967).
On one hand, MPO is a strongly basic protein and can bind to the surface of a negatively
charged cell, potentially allowing for continuous propagation of the antimicrobial
response. On the other, highly reactive products of this system, such as HOCl, act to kill
pathogens during phagocytosis (Klebanoff and Hamon, 1972; Nauseef, 2014). More
recent work indicates that in addition to the direct bacteriolytic action of HOCl, this
reactive species can also precipitate chlorinate lipids production and induce tyrosine
chlorination on proteins to alter the function of these important biomolecules (Hazen and
Heinecke, 1997; Meyer et al., 2017; Stocker et al., 2004).

In sepsis, neutrophil activation results in its adherence to endothelium, chemotaxis,
degranulation and the production of reactive oxygen intermediates in an attempt to
eliminate infiltrating bacteria, but occurs at magnitudes that produce dysfunction and
death of normal cells (Duignan et al., 1986; Regel et al., 1987; Solomkin et al., 1985; Venezio et al., 1982; Wenisch and Graninger, 1995; Zimmerman et al., 1989). Using o-dianisidine-H$_2$O$_2$ analysis of circulating MPO levels, Nikhil et al established MPO enzyme activity in plasma as an indicator of inflammation and onset of sepsis (Bradley et al., 1982; Kothari et al., 2011). To function as a catalytic enzyme, MPO harnesses the major reactive chlorinating species (RCS) from HOCl in equilibrium with $\cdot$OCl and Cl$_2$ to target plasmalogens enriched in the plasma membranes of many cells present in the mammalian cardiovascular system that drives the release of chlorinated species (Ford and Gross, 1989; Harrison and Schultz, 1976; Thukkani et al., 2003). Although the detailed mechanism for MPO-derived 2-chlorofatty acids contributing to human sepsis remains under debate, studies conducted by our research group and others have established an important role for 2-chlorofatty acid (2-ClFA) in sepsis, especially with regard to their role in the development of the acute respiratory distress syndrome (ARDS), consistent with the in vivo data shown in Fig. I-1 (Meyer et al., 2017; Yu et al., 2019). We will expand on this point in the following chapters as it forms the basis of the proposed hypotheses presented in the dissertation.

iv. Cytokines in sepsis

Cytokines play critical roles in orchestrating the rapid and usually effective response of leukocytes to the detection and elimination of invading microorganisms, with little damage to parenchymal cells. In sepsis, cytokine production can become overwhelming, resulting in a cytokine storm that induces damage to the host. It is noteworthy that parenchymal cells and leukocytes express receptors that can recognize and respond to common pathogen proteins through Toll-like receptors (TLRs) and damage-associated
molecular patterns (DAMPs) receptors (Eleftheriadis et al., 2012). The production of major acute innate cytokines involved in local and systemic responses following leukocyte activation via: (1) DAMPs elaborated by injured tissues and (2) Pathogen-associated molecular patterns (PAMPs) arising from microbial infection coupled to TLR (Eleftheriadis et al., 2012). Therefore, any factors that provide a favorable condition for either step could act to stimulate cytokine secretion. For example, well-characterized DAMPs, intracellular proteins released from damaged cells and extracellular proteins that are modified by inflammatory responses activated by sepsis, increase cytokine levels (Schaefer, 2014). Although leukocytes were once thought to be the major source of innate cytokines, parenchymal cells are increasingly recognized for their role in producing these inflammatory mediator cytokines. Thus, one aim of our studies was to examine cytokine expression profiles in both plasma and in peripheral tissues, in our case the mesentery.

The first cells to respond to infection are generally tissue cells of the monocytic lineage, such as fixed tissue macrophages, that become activated secondary to ligation of TLRs or DAMP receptors on cell surfaces (Eleftheriadis et al., 2012). These receptors trigger a cascade of intracellular signals involving transcription factors such as nuclear factor kappa B (NFκB), and result in transcription of early cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), which are themselves pro-inflammatory and able to activate similar pathways as lipopolysaccharides (LPS) present on bacteria (May and Ghosh, 1998; Shalaby et al., 1989). Moreover, TNF-α also mediates the role of granulocyte macrophage-colony stimulating factor (GM-CSF) to facilitate the recruitment of neutrophils to inflammatory sites (Laan et al., 2003). Despite the fact that CLP
promotes the synthesis of pro-inflammatory cytokines in tissue, the production of anti-inflammatory factors, such as IL-10 also occurs in sepsis (Doughty et al., 1998; Friedman et al., 1997; van Der Poll et al., 1997; van der Poll et al., 1995). Additionally, a novel, eosinophil-independent role for IL-5 in promoting a protective innate immune response in sepsis has been demonstrated (Linch et al., 2012).

Chemokines are a family of small secreted proteins that are responsible for modulating multiple aspects of inflammatory responses and host defense (Baggiolini et al., 1994). Many studies have demonstrated several of these to be key mediators in the host immune response to sepsis (Matsukawa et al., 2000; Steinhauser et al., 2000). As an example, chemokine (C-C motif) ligand 5 (CCL5 or RANTES) triggers the unregulated, exaggerated expression of pro-inflammatory cytokines, resulting in increased injury and mortality following sepsis (Ness et al., 2004). In addition, since neutrophils are the first immune cells to migrate into infected tissue sites, an important step in the initiation of an immune response is the synthesis of the neutrophil-recruiting chemokines, such as chemokine (C-X-C motif) ligand 1 (CXCL1 or GRO/KC) and C-X-C motif chemokine 5 (CXCL5 or LIX). Lastly, migration of monocytes from the blood stream across the vascular endothelium is required for routine immunological surveillance of tissues, a response that involves monocyte chemoattractant protein-1 (CCL2 or MCP-1), chemokine (C-C motif) ligand 3 (CCL3 or MIP-1α) and C-X-C motif chemokine 10 (CXCL10 or IP-10), which also activate tissue resident macrophages (Capers Iv et al., 1997; O'Grady et al., 1999). It is noteworthy that sepsis is associated with a time-dependent increase in circulating levels of vascular endothelial growth factor (VEGF) in both animal and human sepsis models, functioning as an important determinant of sepsis.
morbidity and mortality secondary to its action to disrupt microvascular barrier function (Yano et al., 2006).

v. PAI-1 in sepsis

There is strong evidence for interactions between coagulopathy and systemic inflammation in sepsis, particular with regard to upregulation of plasminogen activator inhibitor-1 (PAI-1, also known as Serpine 1) (Esmon et al., 1999). TNF-α, interleukin-1β (IL-1β), and IL-6 upregulate PAI-1 synthesis by endothelial cells (Emeis and Kooistra, 1986) (Mestries et al., 1994), suggesting that this acute phase protein may be an important exacerbating factor in sepsis-induced disseminated intravascular coagulation (DIC) (Pralong et al., 1989). Indeed, plasma PAI-1 levels are markedly elevated during sepsis triggered by gram-negative bacteremia (Brandtzaeg et al., 1990), and high plasma PAI-1 levels correlate to elevated mortality rates and frequencies of multiple-organ failure (Madoiwa et al., 2006). Since the use of PAI-1 inhibitors in LPS treated mice results in attenuated hypercoagulation (Murakami et al., 1997), whereas Serpine 1 deficiency did not influence the outcome of pneumonia initiated by gram-positive Streptococcus pneumoniae (Rijneveld et al., 2003), it has been suggested that for in vivo evaluation of PAI-1 function during sepsis, bacteremia and coagulopathy should be considered separately.

In addition to its well-known anti-fibrinolytic function, PAI-1 has also been noted to participate in other processes such as cell adhesion and migration (Czekay et al., 2011; Declerck and Gils, 2013; Syrovets et al., 2012). In particular, ischemia/reperfusion (I/R) results in elevated levels of PAI-1 and constitute an independent risk factor for ischemic cardiovascular events (Meade et al., 1993; Van Dreden et al., 2009; Vaughan, 2005;
Wiman et al., 2000). Until recently, it was unclear how PAI-1 contributed to ischemic tissue injury independent of its role in coagulopathy. However, recent work has established that PAI-1 participates in the initiation of postischemic inflammatory responses (Praetner et al., 2018).

vi. Mast cells in sepsis

Once released from bone marrow, mast cells (MCs) circulate as immature precursors in the blood, then they home to most tissues of the body, where they mature under the direction of local growth factors (Gurish and Austen, 2012). As part of this maturation process, MCs acquire masses of granules that gradually become filled with a variety of compounds (Pejler et al., 2010; Wernersson and Pejler, 2014) that are released to the exterior in the degranulation process when MCs are activated. Potential mechanisms involved in mast activation are listed as follows: 1) binding of specific antigen to IgE molecules bound to FcεRI on MC surface; 2) complement components; 3) IgG; 4) neuropeptides; 5) pathogen-derived peptides; 6) cell wall products of bacteria (Galli et al., 2005). Release of these preformed mediators, together with activation-induced de novo synthesis of a large panel of additional inflammatory compounds, MC activation can cause an exceptionally powerful inflammatory response. Among the many mast cell derived mediators released during severe sepsis, the secretion of excessive tumor necrosis factor (TNF) may be a major contributor to the pathology (Piliponsky et al., 2010). As another example, MC-derived IL-4 can worsen septic outcomes via reducing the capacity of macrophages to phagocytize bacteria (Dahdah et al., 2014), whereas IL-10 can promote bacterial persistence by its immunosuppressive function (Chan et al., 2013).
MCs-expressed cathepsin C has also been shown adversely affect survival in CLP-induced sepsis (Clair et al., 2004).

**vii. Potential roles for interactions amongst MPO, PAI-1, and mast cells in sepsis**

The exploration of biological effects for chlorinated lipids generated from MPO/HOCl axis that elicit the aforementioned responses *in vivo*, suggest that these lipids may play a critical in mast cell activation (Yu et al., 2019). In localized infection, mast cells impair the phagocytic action of resident macrophages, thereby allowing bacterial proliferation while sparing local recruitment of neutrophils and the sequential release of cytokines that aggravate sepsis (Dahdah et al., 2014). However, as the extent of mast cell activation expands in response to systemic bacterial invasion induced by CLP, survival becomes reduced (Echtenacher et al., 1996; Malaviya et al., 1996; Seeley et al., 2011; Sutherland et al., 2008). In addition, these sentinel cells shed exosomes that contain proteins required for their attachment to endothelium, which in turn facilitates the expression and secretion of PAI-1 from endothelial cells (Al-Nedawi et al., 2005; Armstrong, 1988; Black, 1980; George et al., 1986). The production of PAI-1 in sepsis is also regulated by cytokine release (Healy and Gelehrter, 1994). Given its ability to promote leukocyte/endothelial cell interactions (Praetner et al., 2018), it has been suggested that PAI-1 may contribute to sepsis-induced inflammatory responses and organ dysfunction in patients suffering from severe infection (Healy and Gelehrter, 1994), a notion that has not been experimentally validated.

Recent studies provide compelling evidence that chlorinated lipids have the potential to exert profound effects at the blood-endothelial interface and to contribute to
microvascular changes occurring in sepsis (Yu et al., 2019). As the search for the effect of these lipids that target endothelial cells in the microcirculation has progressed, the concept that the presence of these chlorinated lipids in the circulation can be used as an indicator for severity of disease or clinical outcome in septic patients has emerged. In addition, the fact that chlorinated lipids induce inflammatory changes that are very reminiscent of that accompanying sepsis suggest that MPO inhibition may provide a new avenue for treatment. Some MPO inhibitors can act reversibly by competing for binding at the active site whereas the irreversible inhibitors form strong covalent bonds with iron to block the access for active site (Galijasevic, 2018). Moreover, we propose that other therapeutic approaches targeting downstream sequelae to MPO-dependent chlorinated lipid production (including PAI-1 inhibition with PAI-039 or mast cell stabilization with cromolyn) may be equally or more effective (Dai and Korthuis, 2011; Fay and Korthuis, 2018; Gaboury et al., 1995; Ji et al., 2016).

Research regarding the role of MPO activation and its downstream mediators may provide valuable pathogenic insights as well as guide the establishment of reliable biomarkers for early detection. This dissertation presents work regarding the role of MPO and chlorinated lipids in inducing mast cell activation and PAI-1 formation as mediators of enhanced leukocyte/endothelial interactions in sepsis. In Chapter II, I will present data supporting a role for MPO in sepsis-induced inflammatory responses. In these studies, we employed two mechanistically distinct MPO inhibitors, N-acetyl lysyltyrosylcysteine amide (KYC) and 4-aminobenzoic acid hydrazide (ABAH), to show that inflammatory responses to CLP-induced sepsis can be prevented. Chapter III describes my work in characterizing markers (formation of pro-inflammatory cytokines and chemokines) of the
hyper-inflammatory phase of sepsis are altered, as well as the expression of anti-inflammatory cytokines as markers of the immunosuppressive phase of sepsis in the presence and absence of MPO inhibitor treatment. A scheme focusing on the linkage between MPO activity and chlorinated lipids generation is proposed in an attempt to elucidate the biological role of MPO and its downstream effects in sepsis.

While Chapter III establishes the complex relationships between MPO/HOCl/chlorinated lipids and sepsis, Chapter IV focuses on our search to establish downstream mediators of enhanced MPO activity in sepsis. By application of a PAI-1 inhibitor or mast cell stabilizer, we show roles for PAI-1 and mast cell activation as downstream mediators in CLP-induced leukocyte/endothelial cell interactions. Chapter V summarizes the results and interpretation of these findings and finishes with the expectation that our ongoing studies formulate the basis for embarking on future studies focusing on mediator-based early detection and therapeutic targeting for sepsis.
Figure I-1. Lung wet/dry weight ratio.

Quantitative assessments of the effect of cecal ligation and puncture (CLP)-induced sepsis in rats on the development of pulmonary edema, a major cause of death in septic patients. The first two columns shown above represent the calculated ratios of mean lung wet/dry weight, an indicator for pulmonary edema, for sham CLP (SCLP) and CLP treatment as labeled. A specific MPO inhibitor, N-acetyl lysyltyrosylcysteine amide (KYC, 6 mg/kg, i.p.), was applied 24 hours and 1 hour before the surgery to the same two groups to attain the data in the last two columns. The bar chart summarizes the results as follows: $4.86 \pm 0.08$ for SCLP, $5.14 \pm 0.32$ for CLP, $4.85 \pm 0.09$ for SCLP + KYC, and $4.87 \pm 0.06$ for CLP + KYC, respectively. * indicates $P < 0.05$, ** indicates $P < 0.01$. 
viii. References


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CHAPTER II: ROLE OF MYELOPEROXIDASE IN CECAL LIGATION AND PUNCTURE-INDUCED SEPSIS
i. Abstract

Myeloperoxidase (MPO) is a heme protein stored in the neutrophil azurophilic granules. In addition to serving as one of the main pillars of neutrophil attack, this enzyme may serve as a potentially novel biomarker for sepsis to assist in diagnosing and predicting mortality in sepsis. In biological systems, MPO-derived hypochlorous (HOCl) reacts with the vinyl ether bond of plasmalogens found in membrane lipid compartments to yield α-chlorofatty aldehydes such as 2-chlorohexadecanal (2-ClHDA) and its metabolite 2-chloro fatty acid (2-ClFA). Remarkably, some evidence suggests that either the lethal complications (such as acute respiratory distress syndrome (ARDS)) or 30-day mortality in sepsis was not associated with MPO but was correlated with elevated 2-ClFA levels in sepsis, an observation suggesting a potential direct role of free 2-ClFA in the inflammatory responses induced by sepsis. Indeed, recent studies confirmed that 2-ClHDA and 2-ClFA serve as mediators of the inflammatory responses to sepsis, yet the underlying mechanisms remains unclear. Considering that no chlorinated lipids scavenger is available, and based on the well-established role of the MPO/HOCl/chlorinated lipids axis in inflammatory responses, we hypothesized that treatment with the reversible MPO inhibitor KYC or irreversible inhibitor ABAH would inhibit inflammatory responses induced by cecal ligation and puncture (CLP), a model of polymicrobial sepsis. We observed that these mechanistically distinct inhibitors abrogated CLP-induced inflammatory responses. Specifically, both inhibitors abolished plasma acute lung injury and increased free 2-ClFA levels, leukocyte/endothelium interactions, and mast cell activation in our CLP model. We also found that CLP-induced increases in plasminogen activator inhibitor-1 (PAI-1) levels were also prevented by MPO inhibitor treatment.
These results suggest that MPO may contribute to sepsis-induced 2-CIFA production, leukocyte-endothelium interactions, mast cell activation, and PAI-1 expression. Taken together with our previous work showing that exposing tissues to 2-CIFA in lieu of sepsis causes mast cell activation and leukocyte-endothelial cell adhesive interactions, these observations raise the intriguing possibility that MPO-induced 2-CIFA production may cause the expression of PAI-1 and mast cell activation, to promote leukocyte-endothelial cell interactions in sepsis.
ii. Introduction

Cecal ligation and puncture (CLP)-induced sepsis is currently considered as the gold standard among all experimental animal models. This is because it closely resembles the progression and characteristics of human sepsis and as such has become the most widely used model in sepsis research (Parker and Watkins, 2001; Rittirsch et al., 2008; Wichterman et al., 1980). The model employs bowel perforation with leakage of fecal contents into the peritoneum, which establishes an infection with mixed bacteria coupled with inflammatory mediator release from the necrotic cecum (Ayala et al., 2000; Wichterman et al., 1980). CLP has several other advantages including 1) CLP is relatively easy to produce; 2) the severity of sepsis can be adjusted by the needle puncture size, the number of punctures, or the position of ligation (Baker et al., 1983); 3) most notably, CLP recapitulates the hemodynamic and metabolic phases of human sepsis; 4) the host immune responses with cell types undergoing apoptosis appear to mimic the course of human disease, adding further clinical validity (Ayala and Chaudry, 1996; Hotchkiss et al., 2003). While the CLP model can be adapted to induce sepsis with a range of severity, this advantageous feature also represents a potential major weakness, implying that in CLP-induced sepsis, highly consistent application of needle puncture size and number and position of the ligation must be consistently applied in order to obtain reliable and reproducible results (Singleton and Wischmeyer, 2003).

Sepsis is associated with a wide variety of intense inflammatory responses in the microcirculation that includes 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 (De Backer et al., 2014; Kalogeris et al., 2012; Kalogeris et al., 2016). Sepsis-induced organ failure is multifactorial, involving a complex interplay among invading pathogens, inflammatory cells, and endothelial cells in the microcirculation that produce endothelial barrier disruption and endothelial cell dysfunction (Brown et al., 2006; Cepinskas and Wilson, 2008). Bioactive lipids, generated at the interface of inflammatory cells, neutrophils and endothelium, have been proposed as potential mediators of the pathophysiological sequelae of sepsis and inflammation (Aziz et al., 2013; Graler, 2012; Spite et al., 2009; Teixeira-da-Cunha et al., 2013; Ware et al., 2011). Moreover, these bioactive lipids have also been proposed as potential mediators of the acute respiratory distress syndrome (ARDS), a common sepsis-induced organ failure with high observed mortality rate (Bellani et al., 2016; Fang et al., 2007; Wiedemann et al., 2002) that is characterized by accumulation of protein-rich fluid inside the alveoli that results from damage of the capillary endothelium and alveolar epithelium (Martin, 1999).

Considering the complex molecular pathways of inflammatory responses, in next few chapters, we thus carried out a series of experiments to unveil the mechanisms underpinning modulation at the levels of chlorinated lipids, PAI-1, and mast cells with their unique inhibitors. Sepsis occurs when the body's response to bacterial invasions becomes overexuberant, triggering host immune response that can damage multiple organ systems. Neutrophils are the initial responder, and once activated, release their granule contents, especially myeloperoxidase (MPO). Considering that MPO synthesizes hypochlorous acid (HOCl) and other reactive oxygen species (ROS), neutrophils pose a threat to native cells, causing collateral damage (Klebanoff, 2005; Winterbourn et al.,
Although it is well established that MPO-derived HOCl targets membrane plasmalogens in neighboring endothelial cells leading to the production of 2-chlorohexadecanal (2-CIHDA) and its metabolite 2-chlorinated fatty acid (2-ClFA), their role in diseases characterized by extensive inflammation was not appreciated until recent years (Thukkani et al., 2003; Wacker et al., 2013; Wildsmith et al., 2006). 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 and led to the notion that plasma 2-ClFAs levels may serve as a predictor for the appearance of ARDS. In addition, direct application of 2-CIHDA and 2-ClFA to normal tissues in lieu of sepsis induced inflammatory responses that are very similar to that which occur in sepsis (Meyer et al., 2017; Yu et al., 2019).

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 system in neutrophils (Ford, 2010; Podrez et al., 2000; Podrez et al., 2002a; Podrez et al., 2002b). However, the existence of mechanistically distinct inhibitors of MPO provides a tool to determine if the catalytic activity of this enzyme contributes the pathogenesis of inflammation in sepsis. By correlating protective actions of these inhibitors on sepsis induced 2-ClFA levels and inflammation, we can begin to explore this link. To this end we used two MPO inhibitors, one of which (N-acetyl lysyltyrosylcysteine amide or KYC, Fig. II-1A KYC) exerted reversible inhibition by forming a complex with MPO that competes with MPO substrates in the active site of the enzyme (Galijasevic, 2019) The other inhibitor, 4-aminobenzoic acid hydrazide (ABAH, Fig. II-1B) is oxidized by MPO to a radical species that reduces the enzyme to its ferrous intermediate, thereby allowing
it to interact with either oxygen to allow enzyme turnover, or with hydrogen peroxide to produce irreversible inactivation of the heme center activation site (Furtmüller et al., 2003; Kettle et al., 1997; Ramirez-Duran et al., 2013; Ward et al., 2013).
Figure II-1. Comparison of chemical structures of MPO inhibitors.

Chemical structures of KYC (A) and ABAH (B) as marked.

iii. Materials and methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri. Sprague-Dawley rats (10-14 weeks) acquired from ENVIGO (Indianapolis, IN) were housed in climate controlled, 12:12 hour 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 much lower levels.

Sepsis model

All rats (n=6 in each group, unless otherwise indicated specifically) were anesthetized with a mixture of ketamine (90 mg/kg body weight i.p.) and xylazine (10 mg/kg body weight i.p.). Once showing no flexion of extremity by toe pinch, the abdomen was shaved and, followed by longitudinal skin midline incision (3-4 cm) without penetration into the peritoneal cavity. The abdomen was opened along the linea alba, after which the cecum was identified and exteriorized. After careful dissection of the mesentery of the cecum, without damage the mesenteric blood vessels as shown in Fig. II-2, 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. Experiments were carried out in a modified cecal ligation and puncture model, in which the blood supply for cecum was not fully blocked by placing an 18G 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 instead of ten to twenty percent decline over the first hours after induction of CLP, 100% of animals remain viable for at least six hours after this modified CLP surgery regardless of the degree of ligation (data not shown). Because the degree of injury is reduced by this modified CLP surgery, mesenteric microvascular patency is preserved, enabling in vivo investigation of microcirculatory responses with intravital microscopy.

Cecum were perforated by single through to through puncture midway between the ligation and the tip of the cecum in a mesenteric - to - antimesenteric direction. After removal of needle, a droplet of feces from the penetration holes on both the mesenteric and antimesenteric sides were extruded to ensure puncture patency. In a sham surgery group, the cecum was exteriorized without ligation or penetration. Upon the closure of the abdominal wound with suture, animals were resuscitated by injecting prewarmed normal saline (37 °C; 5 ml per 100 g body weight) subcutaneously between the shoulder blades. 5% lidocaine ointment USP (Fougera Pharmaceuticals Inc.) was placed along the stitching for postoperative analgesia. The rats were then placed in cages at the end of the surgical procedures where the access to water and food is available in cages that, in a temperature-controlled room (22 °C) with 12-h light and dark cycles. All animals were monitored (Rittirsch et al., 2008), before experimental measurements (see below) were obtained 6 hours after CLP or sham CLP (SCLP) surgery.

Tissue samples from the small intestine, mesentery, lung, as well as plasma were collected at the end of experiments for further analysis.

Chemicals and solution compositions
KYC powder was provided by Bill Neumann (Southern Illinois University-Edwardsville) and kept at -20°C until use. KYC was dissolved in 1x PBS (Phosphate Buffered Saline, pH 7.4) and stored as a 3 mg/ml stock at -20°C, for use within two weeks.

ABAH was purchased from Sigma-Aldrich and stored at 4°C. Considering the limited solubility, we performed the solubility experiments with different solvents and concentrations. It turned out that ABAH was dissolved in saline at the maximum concentration of 10 mg/ml after heating in a water bath at 60 degrees for 10 min.

**KYC and ABAH pretreatment**

Pretreated rats with a reversible MPO inhibitor KYC (6 mg/kg; kindly provided by Saint Louis University) 24 hrs (first dose) and 1 hr (second dose) before induction of CLP or SCLP, or with an irreversible MPO inhibitor ABAH (40 mg/kg; Sigma-Aldrich, St Louis, MO) 3 hrs prior to CLP or SCLP surgery as previously described (Forghani et al., 2015; Kim et al., 2016; Reber et al., 2017). The inflammatory responses described below were assessed in six groups (SCLP, CLP, SCLP + KYC, CLP + KYC, SCLP + ABAH and CLP + ABAH) of rats (n=6 except otherwise indicated specifically).

**Immunohistochemistry Staining for MPO in small intestine**

The collected small intestine samples were fixed in 10% (w/v) phosphate-buffered saline (PBS)-buffered formaldehyde and embedded in paraffin. Following dewaxing, endogenous peroxidase was quenched with 3% (v/v) hydrogen peroxide for 5 minutes. The slides were incubated in 5% (v/v) bovine serum for 20 minutes to block non-specific binding. Sections were then incubated with anti-MPO antibody (ab9535, 1:50 in PBS, Abcam, MA) for 60 minutes. Samples were rinsed in wash buffer and incubated with
detection system for 30 minutes. Slides were applied with 3, 3’-diaminobenzidine (DAB) substrate and stained with Mayer’s hematoxylin for 30 seconds, then dehydrated in graded alcohol and xylene. Immuno-histochemical images were collected using an Olympus microscope (40x) and image-pro plus software version 5.1.2.59. For graphic display of staining analysis, the immune reactivity was expressed as the mean total number of positive staining cells in each filed.

*Plasma lipid extraction for free 2-CIFA*

From all of the SCLP, CLP, and groups pretreating with KYC or ABAH experiments, whole blood was collected, from that plasma was well prepared. 25 µl of plasma was added to a 16x100-mm glass culture tube containing the dried internal standard, following the addition of 475 µl saline and vortex. Sequentially add 2.5 ml of Dole reagent (made fresh; isopropanol/heptane/1 M H₂SO₄, 40/10/1, v/v/v), 1.5 ml of heptane, and 1 ml of water and vortex again. Centrifuge at 500g for 2 minutes to collect the upper phase into a clean 16x100-mm ScrewTop tube. For the lower phase, re-extract with 2 ml of heptane, vortex, and centrifuge at 500g for 2 minutes. Collect the upper phase and combine it in a glass tube with the previously collected upper phase. Dry the heptane extracts under N₂ and resuspended in 150 µl of methanol/water (85/15, v/v) containing 0.1% formic acid. Vortex vigorously and transfer the sample to an autosampler vial with an insert (Dole, 1956; Wacker et al., 2013).

*Intravital Microscopy*

As in our previous studies (Yu et al., 2019), 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 a prewarmed saline (37°C). Body temperature was maintained between 36.5-37.5°C by use of a thermistor-controlled heat lamp. The Plexiglas board was mounted on the stage of an inverted microscope (Eclips TE2000; Nikon) and postcapillary venules approximately 20-50 µm in diameter in mesentery were observed, typically with 20x magnification (Zuidema MY, 2015). 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-1953MD; Sony) and recorded on a DVD recorder (DMR-E50; Panasonic) or captured through Metamorph software version 7.8. (Nashville, TN) A time-date generator (WJ810, Panasonic) displayed this function on the monitor.

Albumin Leakage

FITC-labeled albumin (Sigma-Aldrich, St Louis, MO) was used to evaluate the albumin leakage across the post-capillary venule in rat mesenteric circulation. To quantify albumin leakage across mesenteric venules, 50 mg/kg of labeled albumin was administered intravenously to the animals 15 minutes before the recording. Rat mesentery was exteriorized and prepared for intravital microscopy as described above. Six hours after surgery, 10 single unbranched post-capillary 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 5 regions of interest (25 µm in diameter) within the venules (Iv) and in the perivenular interstitium within 10-50 µm of the venular wall (Ip) were measured using Metamorph software version 7.8. Albumin leakage, estimated by Ip/Iv at each pair of corresponding circles was designated as the indicator of albumin leakage (Zuidema MY, 2015). 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.

*Lung Histology*

Some CLP and SCLP rats were secured to a dissecting board, followed by a ventral midline incision into abdomen to expose the peritoneal cavity and the xiphoid process. Scissors were then used to cut the ribs on each side of the sternum at about the mid-level of the body of the ribs to expose the thoracic cavity. Heart and other organs were separated from the trachea and lungs carefully to expose the trachea, which was dissected, and a short cross-sectional incision was made in the upper part. An 18G blunted needle connected to a 3-5 ml syringe containing 10% formalin is gently threaded into trachea that, then ligated with 4-0 black silk suture (Roboz Surgical, MD) to hold the needle in place. The lungs were gently inflated with formalin until a full expansion to a normal level as expected to fill the chest cavity. Ligation of the trachea to hold the trachea for keeping the lung expanded, then the organ mass including all lung lobes and trachea was placed in formalin for 48-72 hours. Following tissue processing and embedding, the lungs were sampled as appropriate to the particular study (Morton and Snider, 2017). Sections were deparaffinized in xylene, rehydrated in graded alcohol concentrations and stained with hematoxylin and eosin. Stained sections were then dehydrated and permanently mounted with glass coverslips.

*Evaluation of degree of CLP-induced lung injury*

The lung was sectioned at a thickness of 5 μm, placed on glass slides, and stained with hematoxylin-eosin. The degree of lung injury was examined, and the specimens was
graded by acute lung injury (ALI) score (Kim et al., 2008) based on (a) alveolar capillary congestion, (b) intra-alveolar hemorrhage, (c) infiltration or aggregation of neutrophils in the airspace or the vessel wall, and (d) 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%) (Imanaka et al., 2001). The degree of ALI was assessed by the sum of scores for items 0 to 16 in ten randomly selected high-power fields (HPFs, 400x). The average of the sum of each field score was compared among groups (Kim et al., 2008).

**Leukocyte-platelet-endothelium interactions**

The procedures and fluorescent labeling of leukocytes and platelets are similar to what we have used previously in our lab (Dai et al., 2017; Zuidema MY, 2015). First of all, 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. Rolling cells are defined to be those passing a line drawn onto the computer monitor at right angles to the vessel under observation at a velocity significantly slower than the centerline velocity and were expressed as rolling cells per 30 seconds. Adherent leukocytes or platelets (differentiated by size) were defined as those cells that remain adherent to the venular wall for at least 30 seconds. The numbers of adherent cells were expressed per mm² surface area.
Mast cell activation

Ruthenium red (EMD Millipore, MO) was dissolved to 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 (Steiner et al., 2003). Exteriorized mesentery was superfused with ruthenium red (0.001%) for 15 minutes, after which 10 fields were randomly chosen for quantifying the number of activated mast cells in each field.

Multiplex analysis of plasma PAI-1

Plasma were 2x diluted in 1x PBS solution. The multiplexing analysis was performed using the Luminex™ 100 system (Luminex, Austin, TX, USA) by Eve Technologies Corporation (Calgary, Alberta). Four markers were simultaneously measured in the samples using a MILLIPLEX Rat Vascular Injury Panel 1, 4-plex kit (Millipore, St. Charles, MO, USA) according to the manufacturer's protocol. The 4-plex consisted of total plasminogen activator inhibitor (tPAI), Caveolin-1, connective tissue growth factor (CTGF), tissue inhibitor of metalloproteinase-1 (TIMP-1). The assay sensitivities of these markers range from 0.086-1.928 ng/mL for the 4-plex. Individual analyte values are available in the MILLIPLEX protocol.

Data analysis and statistics

MetaMorph (Nashville, TN) was used to estimate the albumin leakage, quantify leukocyte rolling and adhesion, platelet rolling and adhesion, and mast cell activation. All data were shown as sample mean ± standard error of the mean; n represents the number of rats in each set of experiment. Multiple comparisons were initially evaluated by ANOVA followed by Fisher’s LSD test to compare the values among SCLP, CLP, and
groups pretreated with MPO inhibitors. Graphpad Prism 6.0 was used to generate all statistical values. A $p$ value of 0.05 or less is considered to be the level of statistical significance.
Figure II-2. Schematic illustration of CLP.

Characterized positions of cecal ligation to induce mid-grade (medium ligation) or high-grade sepsis (large ligation). The mesenteric vessels (depicted in red in the yellow mesentery) near the region of the cecum to be ligated were carefully dissected free so that blood flow was not impaired after placement of needle and tying of the cecal ligature.
iv. Results

In the first series of experiments, we quantified neutrophil infiltration by assessing MPO expression using an immunobiological approach we employed in earlier work (Yu et al., 2019). 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. II-3A. CLP induced a marked increase in MPO staining, an effect that was abrogated by pretreatment with either of the MPO inhibitors (Fig. II-3B).

Although a number of studies have provided evidence indicating that MPO is an indicator of inflammation in sepsis (Kothari et al., 2011; Schrijver et al., 2017), including our present and previously published work (Yu et al), the mechanisms whereby MPO may contribute to the proinflammatory effects of sepsis remain unclear. However, it has been suggested that MPO-derived 2-ClFA may play a role in human sepsis mortality via induction of ARDS (Meyer et al., 2017). To directly address this question, we evaluated the effect of MPO on the generation of free 2-ClFA in plasma obtained from septic rats that were treated with the MPO inhibitors KYC or ABAH or vehicles. Of note, the molecular species of 2-ClFA that we analyzed is 2-ClFA (18:0 Cl), 2-chlorostearic acid. As shown in Fig. II-4, 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 lessened about 1/2 of free 2-ClFA (18:0 Cl) generation and ABAH lowered around 1/3 compared to the initial levels in SCLP and CLP groups. Fig. II-4 summarized these data in a bar graph.
A reduction in the restrictive properties of the endothelial barrier in postcapillary venules is one of the earliest signs of microvascular dysfunction in inflammatory conditions such as sepsis (Granger and Korthuis, 1995; Kalogeris et al., 2011, 2012). In this study, we confirmed our earlier results (Yu et al) showing that CLP-induced sepsis resulted in an increase in venular protein leakage (Figure II-5). Sham CLP surgery causes very little albumin leakage, resulting in a permeability ratio $I_p/I_v$ of $0.79 \pm 0.11$, $n=6$. On the other hand, CLP was associated with a significant increase in albumin leakage ($I_p/I_v = 1.15 \pm 0.02$, $n=6$). A significant new finding is that this CLP-induced increase was abolished by treatment with the MPO inhibitor KYC ($I_p/I_v = 0.72 \pm 0.02$, $n=6$), but was without effect in SCLP ($I_p/I_v = 0.73 \pm 0.02$, $n=6$) (Figure II-5).

Given our data demonstrating that MPO is involved in CLP-induced venular protein leakage, which is the hallmark of ARDS, we next postulated the possibility that MPO engages in CLP-induced ARDS by mediating neutrophil activation. Fig. II-6A presents representative histologic images of acute lung injury induced by CLP vs 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 six hours after induction of CLP and was largely abrogated by pretreatment with KYC or ABAH. Injury was quantified using an injury scoring system, with results presented in Fig. II-6B: SCLP group ($4.02 \pm 0.15$, $n=6$) versus CLP group ($10.13 \pm 0.29$, $n=6$). The increased injury score induced by CLP was largely prevented by pretreatment with either MPO inhibitor: $4.73 \pm 0.24$ for SCLP + KYC, $n=6$; $4.77 \pm 0.18$ for CLP + KYC, $n=6$; $4.88 \pm 0.13$ for SCLP + ABAH, $n=8$; and $5.14 \pm 0.25$ for CLP + ABAH, $n=8$. 

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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 (Yu et al). Pretreatment with either KYC or ABAH completely prevented CLP-induced leukocyte rolling (Fig. II-7) and adhesion (Fig. II-8). Taking together, these data indicate that the effect of CLP-induced sepsis to increase leukocyte rolling and adhesion is dependent of MPO. Furthermore, our results with regard to the effects of KYC and ABAH on plasma free 2-ClFA suggests that these chlorinated lipid species may contribute to these enhanced leukocyte-endothelial interactions, as we previously proposed. (Yu et al., 2019).

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 (Bone, 1991). 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 post-capillary venules. As shown in Fig. II-9, a large increase the numbers of rolling platelets upon cecal ligation and puncture surgery can be readily discerned in column 1 and 2, 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. II-10).
Because mast cell activation occurs in sepsis and following provocation with 2-CIFA and promotes leukocyte rolling and adhesion (Meyer et al., 2017; Yu et al., 2019), we next sought to determine whether this response to CLP could be abrogated by pretreatment with the MPO inhibitors. Fig II-11 shows a summary of these experiments. CLP elicits mast cell activation by ~ 5-fold compared to SCLP group. This response to CLP was abolished by pretreatment with KYC and ABAH (Fig. II-11).

We next addressed the hypothesis that CLP may induce an increase in plasma levels of PAI-1. Indeed, data presented in Fig. II-12 shows that PAI-1 concentration in plasma of CLP rats was increased relative to SCLP. Pretreatment with ABAH but not KYC prevented CLP-induced increase in plasma PAI-1.
Figure II-3. Immunohistochemical staining for MPO in small intestine.

(A) Representative immunohistochemistry (IHC) images showing MPO staining is markedly elevated in animals subjected to CLP vs SCLP. The orange arrowhead indicates positive staining (brown) cells. Scale bars=50 μm. (B) In this panel, we present the number of MPO positive cells obtained from 10 fields at a magnification of 40x. IHC staining showed that a significant increase in the number of MPO positive cells (15.45 ± 0.93, n=6) was induced by CLP challenge compared to 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). **, p < 0.01.
Figure II-4. Plasma free 2-ClFA analysis in response to MPO inhibitors pretreatment.

Free 2-ClFA (18:0 Cl) in plasma. CLP induced a modest increase of 2-ClFA relative to the SCLP group (1.67 ± 0.39 for CLP; 1.45 ± 0.27 for SCLP. n=6). KYC pretreatment was associated with significantly decreased plasma free 2-ClFA levels in both groups [SCLP + KYC group (0.51 ± 0.12, n=6), and CLP + KYC group (0.45 ± 0.1, n=6)]. As expected, regulation of MPO with ABAH also dramatically suppressed 2-ClFA production in plasma, as listed here: (0.81 ± 0.1, n=8) for SCLP + ABAH, and (1.02 ± 0.1, n=8) for CLP + ABAH. *, p < 0.05 and **, p < 0.01.
Figure II-5. Comparisons of permeability between SCLP and CLP groups in the absence and presence of pretreatment with the MPO inhibitor KYC.

CLP was associated with a significant increase of albumin leakage, an effect that was abolished by pretreatment with KYC (n=6). ** indicates $p < 0.01$. 
Alveolar congestion
Intra-alveolar hemorrhage
Neutrophils infiltrating in the airspace and vessel wall
Thickness of alveolar wall/hyaline membrane formation
Figure II-6. Histologic evidence of lung injury induced by CLP is reduced by pretreatment with MPO inhibitors.

Histopathological images (Panel A) and quantification of acute lung injury (ALI) scores from these images (Panel B). (A) CLP-induced lung injury group showed typical findings of lung injury, 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 ABAH, images in middle and bottom). (B) Summary of the ALI scores in different groups as indicated. n=6 in first four groups, while n=8 in SCLP + ABAH group and CLP + ABAH group. **, $p < 0.01$. 
Figure II-7. Effects of KYC and ABAH on CLP-induced leukocyte rolling.

Compared to SCLP (1006.34 ± 24.19), CLP produced a marked increase in the number of rolling leukocytes (2404.42 ± 91.40). KYC pretreatment abolished this CLP-induced increase (1121.77 ± 48.32) and was without effect in SCLP (1101.68 ± 43.38). Similar results were obtained in rats treated with the irreversible MPO inhibitor ABAH (CLP + ABAH: 1248.51 ± 82.89; SCLP + ABAH: 1282.45 ± 119.45). n=6. **, p < 0.01.
Figure II-8. KYC and ABAH inhibit CLP-induced leukocyte adhesion.

Cecal ligation and puncture was associated with a significant increase in the numbers of firmly adherent leukocytes on postcapillary venules (260.79 ± 40.1) in CLP compared to SCLP group (143.42 ± 30.37). KYC prevented this response in CLP animals (number of adherent leukocytes averaged: 141.08 ± 22.52 in SCLP + KYC and 152.29 ± 26.36 in the CLP + KYC group). Similar results were obtained in animals treated with ABAH (98.46 ± 24.36 for SCLP + ABAH; 88.32 ± 22.37 for CLP + ABAH). n=6. * indicates p < 0.05, and ** indicates p < 0.01.
Figure II-9. Differential effectiveness of MPO inhibition with KYC versus ABAH on CLP-induced platelet rolling.

Pretreatment with KYC, but not ABAH, abolished CLP induced platelet rolling (number of rolling platelets averaged: 331.1 ± 99.66 for SCLP, 727.46 ± 159.71 for CLP, 274 ± 110.51 for SCLP + KYC, 506.09 ± 66.88 for CLP + KYC, 733.52 ± 198.99 for SCLP + ABAH and 1037.83 ± 220.86 for CLP + ABAH. n=6. *, p < 0.05; **, p < 0.01.
Figure II-10. KYC and ABAH abrogate platelet adhesion in sepsis.

A comparison of data presented in the first two columns indicate that CLP induced an increase in the number of firmly adherent platelets (to 79.76 ± 14.9 in the CLP group from 26.97 ± 12.45 in the SCLP group). Pretreatment with a reversible MPO inhibitor KYC or irreversible inhibitor ABAH prevented this increase (42.74 ± 8.76 in the CLP + KYC group and 34.68 ± 16.77 in the SCLP + KYC group; 38.77 ± 27.58 in the CLP + ABAH group and 10.43 ± 7.77 in the SCLP + ABAH). n=6. * indicates p < 0.05.
Figure II-11. CLP-induced mast cell activation is abolished by pretreatment with MPO inhibitors, KYC and ABAH.

CLP induced a large increase in the numbers of activated mast cells relative to SCLP. This effect was abrogated by pretreatment with KYC or ABAH (average number of activated mast cells: 3.72 ± 1.28 for SCLP, 5.87 ± 0.59 for SCLP + KYC, and 4.15 ± 0.27 for SCLP + ABAH; 21.32 ± 1.31 for CLP, 6.33 ± 0.91 for CLP + KYC, and 4.7 ± 0.3 for CLP + ABAH. n=6. **, p < 0.01.
Figure II-12. CLP increased plasma concentrations of total PAI-1 are abolished by ABAH but not KYC.

Animals were treated with KYC or ABAH prior to the SCLP or CLP to determine the effects of MPO inhibition on the increase in plasma PAI-1 levels induced by CLP. CLP increased plasma total PAI-1 (3.75 ± 0.69, n=6) relative to SCLP (1.3 ± 0.29, n=5). The CLP induced increase in plasma PAI-1 was abolished by pretreatment with ABAH (0.51 ± 0.2, n=8) but not KYC (3.53 ± 1.31, n=5). By contrast, in SCLP groups, ABAH indeed restricted total PAI-1 circulating in plasma (0.17 ± 0.04, n=8) while not much effect was noticed from KYC usage (1.08 ± 0.27, n=6). ** refers $p < 0.05$. 
v. Discussion

In this study, we showed that CLP caused neutrophil infiltration into the tissue space, enhanced the generation of the proinflammatory mediator 2-ClFA, induced increases in mesenteric venular protein leakage, 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 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 onset of sepsis (Kothari et al., 2011). 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 (Meyer et al., 2017). In addition, we presented evidence that in intact rat mesentery, exogenous chlorinated lipids including 2-ClHDA and its metabolite 2-ClFA elicit inflammatory responses in microcirculation that strongly mimic those that characterize sepsis (Yu et al., 2019). Here, we will first discuss the direct role of chlorinated lipid species generated in sepsis based on our current understanding of MPO/HOCl/chlorinated lipids 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. Finally, our results suggest that activation of mast cells and secretion of PAI-1 may serve as downstream mediators of the
inflammatory responses elicited by MPO-derived chlorinated lipids, which will be the subject of subsequent studies presented in this dissertation.

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 (Galijasevic, 2019), recent work has focused on design new MPO inhibitory reagents that exert less toxicity and resist oxidation. KYC is one of these newer generation of competitive MPO inhibitors wherein tyrosine moieties out-compete Cl\(^-\) or NO\(_2\)- for binding to prevent MPO from generating HOCl and •NO\(_2\), conferring specificity in targeting MPO as an inhibitor (Zhang et al., 2013a). 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 (Rymaszewski et al., 2014; Zhang et al., 2016; Zhang et al., 2013b). Being mechanistically distinct from and structurally unrelated to KYC, we also employed the irreversible inhibitor of MPO (Malle et al., 2007), 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 (Forghani et al., 2012). Indeed, the complete inactivation of MPO by ABAH is possible only in the presence of the high concentrations of hydrogen peroxide, which occurs when neutrophilic NADPH oxidase is activated (Galijasevic, 2019). This is based on the observations that ABAH inhibited HOCl production by MPO by up to 90%, an effect that
was abolished by concomitant administration of superoxide dismutase (Kettle et al., 1995).

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 hrs after induction of sepsis. These observations 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-driven HOCl production, which in turn may act as inflammatory mediators to elicit leukocyte-endothelial interactions (Meyer et al., 2017; Yu et al., 2019). 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. II-4. 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 stream of flowing blood. The significant reduction of free 2-ClFA (18:0 Cl) provides clues for the possible similar mechanisms of KYC and ABAH in the formation of 2-ClFA (18:0 Cl), a more related indicator of sepsis as shown in Fig. II-4.

Disruption of microvascular barrier integrity is amongst the earliest detectable responses to sepsis, an effect largely attributed to sepsis-induced cytokine release (Hansen et al., 2011; Rivers et al., 2001). Our results revealed that CLP induced a significant increase in mesenteric venular albumin leakage (Fig. II-5), an effect that was completely prevented
by treatment with the MPO inhibitor KYC. This result suggests that sepsis-induced MPO activity either results in direct injury to the microvascular barrier to promote protein leakage or participates in the downstream generation of proinflammatory mediators that act to disrupt this barrier, a concept consistent with that suggested by Hartman et al (Hartman and Ford, 2018).

Bioactive chlorinated lipids have been implicated as potential mediators of ARDS, a devastating complication of sepsis that results in extremely high mortality (Bellani et al., 2016; Fang et al., 2007; Kim and Hong, 2016; Wiedemann et al., 2002). Although the pathologic events contributing to the development of ARDS occurs in septic subjects continues to evolve, it is clear that pulmonary barrier dysfunction secondary to neutrophil recruitment and activation and excessive free radical production (Gadek and Pacht, 1996) are 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, CLP produced alveolar congestion, intra-alveolar hemorrhage, neutrophil infiltration, and increased thickness of the alveolar wall, effects that were abolished by pretreatment with either MPO inhibitor. Taken together with our observations that MPO inhibition prevents the increase in plasma 2-ClFA levels and venular protein leakage, these data support an important role for MPO, and perhaps 2ClFA, in the development of pulmonary dysfunction induced by CLP.

Previous work from our group indicated that 2-chlorinated palmitic acid (2-ClPA), leads to mobilization of Weibel-Palade bodies and release of angiotensin 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 (Meyer et al., 2017; Yu et al.,
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-ClPA. Importantly, administration of KYC and ABAH prior to induction of CLP prevented these sepsis-induced adhesive interactions, except that ABAH failed to prevent platelet rolling (Fig II-7-10). 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 (Eppihimer et al., 1997; Kanwar et al., 1998; Kanwar and Kubes, 1994a, b). In earlier work (Yu et al., 2019), we showed that chlorinated lipids elicit mast cell activation. Based on 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 support this hypothesis in that both MPO inhibitors ( KYC or ABAH) prevented mast cell activation in our septic model. In Chapter IV, we present data indicating that treatment with a mast cell stabilizer prevents CLP-induced leukocyte rolling and adhesion. Taken together, our data is 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 II-13 presents a summary hypothesis diagram which integrates these responses to CLP.

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 (Al-Nedawi et al., 2005). Indeed, owing to its anti-fibrinolytic actions, PAI-1 may play a role in disseminated intravascular coagulation in sepsis (Madoiwa et al., 2006). In addition, recent work has shown that I/R causes immobilization of PAI-1 secondary to binding to cell surface glycosaminoglycans on neutrophils. This binding provokes affinity changes in β2 integrin on rolling neutrophils to facilitate the transition to stationary neutrophil adherence and subsequent transmigration (Kozlova et al., 2015; Spijkers et al., 2005). In addition, PAI-1 participates in the regulation of vascular permeability (Nagai et al., 2005; Xu et al., 2010), either directly or by its effect to recruit neutrophils (DiStasi and Ley, 2009; Praetner et al., 2018). MPO inhibition with ABAH prior to CLP abolished increases in plasma PAI-1 levels that normally occurs in sepsis (Fig. II-12). 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 doses are required to block this particular effect of MPO, as discussed above. In Chapter IV of this dissertation, we employ a PAI-1 inhibitor approach to provide more direct support for a role for this antifibrinolytic and proinflammatory mediator in CLP-induced sepsis.
Although our data clearly implicates 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 is consistent with the scheme shown in Figure II-13, which may be used as a guide for future studies that require additional work to substantiate or refute. Our data strongly support the concept that CLP-induced MPO activity is required for increased neutrophil emigration, plasma levels of 2-ClFA, venular albumin leakage, 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 pharmacologic inhibitor approaches in Chapter IV. Moreover, we believe that examination of the changes in cytokine expression patterns in response to CLP that are described in the next chapter (Chapter III), together with the molecular insights regarding the role of the MPO/HOCl/2-ClFA in CLP provided in the present chapter should pave the way for early detection and intervention of sepsis.
Figure II-13. Schematic view explaining the effects of MPO inhibitors on downstream effectors.

Our results indicate that MPO inhibition prevents CLP-induced production of 2-CI FA, PAI-1 expression, mast cell activation, leukocyte and platelet adhesive interactions, neutrophil infiltration, and venular protein leakage. Our observations are consistent with the scheme depicted above, but additional work, outlined in Chapters III and IV provides additional support for linkages depicted above. Dashed arrows represent further investigation in future studies.
vi. References


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CHAPTER III: MYELOPEROXIDASE INHIBITION ATTENUATES
THE EXPRESSION OF PROINFLAMMATORY MEDIATORS IN A
CECAL LIGATION AND PUNCTURE MODEL OF SEPSIS
i. Abstract

Consistent with a hyperinflammatory state in the early stages of sepsis, numerous pro-inflammatory cytokines and chemokines have been shown to accumulate in plasma and tissues, which is associated with neutrophil infiltration, as reflected by increased tissue content of myeloperoxidase (MPO). While these inflammatory mediators act in concert to generate the host response towards microorganisms, overexuberant expression in sepsis is thought to contribute to bystander injury in parenchymal cells. While this suggests that these mediators could be targeted for inhibition to limit this injury, most human trials have failed presumably because only a single mediator was selected as a target for intervention. Effective therapy may require development of interventions that target multiple mediators in sepsis or identify upstream mediators for their production. Our recent work (Chapter II) indicates that MPO inhibition reduces the formation of proinflammatory chlorinated lipids, venular protein leakage, acute lung injury, leukocyte and platelet adhesive interactions, mast cell activation, and total plasminogen activator inhibitor-1 (PAI-1). The effectiveness of MPO inhibition in abrogating all these responses suggests the possibility that MPO may play a role in the elaboration of cytokines and chemokines implicated in the pathogenesis of tissues injury in sepsis. To address this issue, we employed two structurally unrelated and mechanistically distinct MPO inhibitors prior to induction of a septic state cecal ligation and puncture (CLP) on the expression profile of a number of cytokines and chemokines in plasma and/or tissues. The results of these studies indicated that both inhibitors effectively reduced CLP-induced increases in a number of cytokines (e.g., interleukin-1α (IL-1α), IL-10, and tumor necrosis factor-α (TNF-α)) and chemokines (e.g., macrophage inflammatory
proteins-1α (MIP-1α, CCL3), monocyte chemoattractant protein-1 (MCP-1, CCL2), C-C Motif Chemokine Ligand 5 (RANTES, CCL5), C-X-C motif ligand 5 (LIX, CXCL5), IFN-γ-inducible protein 10 (IP-10, CXCL10) and granulocyte-macrophage colony stimulating factor (GM-CSF)) in plasma and/or mesenteric tissue. Collectively, these data identify potential contribution of MPO/HOCl system to initiate tissue and plasma cytokine/chemokine in CLP-induced sepsis.
ii. Introduction

Cytokines are glycoproteins that regulate functions of the immune system (Holdsworth and Gan, 2015). Additionally, as a group of secreted proteins within the cytokine family whose generic function is to induce cell migration, the “chemotactic cytokines” or “chemokines” are involved in leukocyte chemoattraction and trafficking of immune cells to locations throughout the body (Walz et al., 1987; Yoshimura et al., 1987).

While cytokines are produced and act locally, they can enter the systemic circulation in quantities sufficient to invoke inflammatory responses at distant sites, thereby contributing to the systemic inflammatory response syndrome (Slifka and Whitton, 2000; Waage et al., 1989). Whether expressed in tissue or the circulation or both, these mediators act in concert to direct and amplify the generation of appropriate patterns of defense to combat microbial threats (Gabay and Kushner, 1999).

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 their migration into the tissues. While transmigration allows these infiltrating leukocytes to target their cytotoxic arsenal on invading bacteria, overexuberant release of reactive oxygen species and hydrolytic enzymes can produce bystander injury in parenchymal cells, producing organ injury, dysfunction and/or cell death (Gabay and Kushner, 1999; Luster, 1998; Ward, 2012). In light of these observations, numerous studies have evaluated the utility of plasma cytokines as biomarkers of sepsis severity and to direct therapy with immune modulating drugs (Oberholzer et al., 2005). Indeed, over the past thirty years, there have been over 100 clinical trials to evaluate the effectiveness of agents that suppress specific mediators of...
inflammatory responses to sepsis, but failed to reduce the mortality from sepsis (Boomer et al., 2011; Hotchkiss and Opal, 2010; Riedemann and Ward, 2003). As a consequence, *Surviving Sepsis Guidelines* were developed and indicated that “No recommendation can be given for the use of these markers to distinguish between severe infection and other acute inflammatory states” (Dellinger et al., 2013). Three main reasons were presented to support this conclusion including: (1) overinterpretation of the value of the sepsis models employed in preclinical studies, (2) the extent to which a given sepsis model mimics human sepsis, (3) the number of cytokines involves and the complexity of their interactions (Dyson and Singer, 2009; Marshall et al., 2005).

In the current study, we demonstrate that in our modified sepsis model, CLP increases the elaboration of pro-inflammatory cytokines (IL-1α, TNF-α, and GM-CSF), anti-inflammatory cytokines (IL-5 and IL-10), chemokines (MIP-1α, MCP-1, RANTES, LIX, IP-10, but not GRO/KC), and a growth factor (VEGF). Our data also demonstrate that the expression of these mediators is dependent on MPO. We therefore propose a potential relation featuring a novel regulating mechanism between MPO-derived free 2-ClFA and cytokine/chemokine/VEGF release in sepsis.
iii. Materials and methods

Animals and sepsis model

See materials and methods in Chapter II.

KYC and ABAH pretreatment

See materials and methods in Chapter II.

Chemicals and solutions

The standard 1x working RIPA buffer contains (in mg) 16 protease inhibitor, 2.7 C₈H₁₀FNO₂S.HCl, 2.25 NaF, 6.25 Na₄P₂O₇ and 50 ul Na₃VO₄.

Plasma preparation

Rats (5-8 in each group, see legends to each figure for number of animals in each group) were anesthetized with a mixture of ketamine (90 mg/kg body weight i.p.) and xylazine (10 mg/kg body weight i.p.) and monitored periodically for depth of anesthesia. After shaving the thorax and abdomen, and “Y”-shaped incision through the abdominal wall was performed, followed by putting aside of the intestines. The liver was retracted to expose the posterior vena cava (between the kidneys). Using a syringe coated with 0.5 M sterile EDTA up in a syringe, we collected blood from the exposed 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 1000 x g for 10 minutes at 4°C within 30 minutes of blood collection (Sorvall ST 40R Centrifuge, Thermo Fisher Scientific, Hampton, NH). Collected plasma was immediately decanted and diluted 2-fold using 1x PBS at pH~7.4. Samples of this diluted plasma were aliquoted into capped vials and stored at -80°C until analysis.
Mesentery preparation

After performing the “Y”-shaped incision in the abdomen wall, the xiphoid process, sternum and diaphragm were opened with scissors to expose the heart. An 18-gauge needle attached to a peristaltic pump via silicon tubing (MINIPULS 3 Peristaltic Pumps, Gilson, Middleton, WI) was inserted into the left ventricle near the apex. Blood was then flushed from circulation by rapid ventricular perfusion (12 mL/min) with ice-cold (4°C) saline containing 20 U per milliliter of heparin (Heparin Sodium Injection, Novaplus, Schaumburg, IL). Once perfusion was initiated, the right atrium was incised to allow drainage. Immediately after flushing the circulation, the mesentery attached to the small intestine was removed, divided at the middle of the tissue into two parts, saving the half closest to the cecum (ileum and a portion of the jejunum), which was snap frozen in liquid nitrogen and saved at -80°C for cytokine/chemokine analysis.

Protein concentration

Homogenization: Using a frozen mortar and pestle, the frozen mesenteric samples were homogenized in liquid nitrogen. The resulting fine powder was transferred into a 1 ml tube with bottom immersed in liquid nitrogen. 1 ml/200 mg tissue of RIPA buffer (see chemicals and solution compositions) was added to the tube and vortexed violently until there were no aggregates.

Sonication: After placing the vortexed mesenteric sample tube on ice, the tip of a sonicator (Vibra-cell, Sonics, Newtown, CT) was then placed into the middle of the tube, being careful to avoid touching any surface of the tube. The sample was then sonicated twice for 10 seconds at a time, with 10 seconds of no sonication in between.
Once tissues are homogenized and sonicated as described above, the tubes were then centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was transferred to a new tube. 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 samples at -80°C until analysis.

*Multiplex analysis of cytokines*

In this study, we used Luminex xMAP technology for multiplexed quantification of 27 rat cytokines, chemokines, and growth factors in plasma and mesentery, respectively. The multiplexing analysis was performed using the Luminex™ 100 system (Luminex, Austin, TX, USA) by Eve Technologies Corporation (Calgary, Alberta). Twenty-seven markers were simultaneously measured in the samples using a MILLIPLEX Rat Cytokine/Chemokine 27-plex kit (Millipore, St. Charles, MO, USA) according to the standard protocol from manufacturer. The 27-plex consisted of G-CSF, Eotaxin, 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 0.3-30.7 pg/mL for the 27-plex. This analysis was carried out in the same six groups descripted in Chapter II.

*Data analysis and statistics*

Plasma cytokine contents (pg/ml) = Individual analyte values x 2

Mesentery cytokine contents (pg/mg protein) = Individual analyte values/protein content.
Multiple comparisons were performed by using two-way ANOVA with Fisher's LSD test as a post hoc test. Graphpad Prism 6.0 was used to generate all statistical values. All data were shown as sample mean ± standard error of the mean (SEM); n represents the number of rats in each set of experiment. A p value of 0.05 was considered statistically significant and the error bars represent SEM in all figures.
iv. Results

Pro-inflammatory cytokines (IL-1α, TNF-α and GM-CSF) and MPO accumulate in tissues after CLP

We recently demonstrated that tissue levels of MPO, a relatively specific indicator for neutrophil infiltration (Guo and Ward, 2005), increase in the small intestine after CLP (Fig. II-3, Chapter II). Because inhibition of this enzyme proved to be very effective in reducing venular albumin leakage, 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 chlorinated lipids that by themselves produce a similar pattern of inflammatory responses as sepsis, supports the feasibility of this postulate. The data presented in Fig. III-1 shows MPO inhibition decreased CLP-induced interleukin 1α (IL-1α) in mesentery.

A similar pattern of response was noted with regard to CLP-induced increases in another important cytokine, tumor necrosis factor alpha (TNF-α), which has been implicated as a primary inflammatory mediator in endotoxin shock (Royall et al., 1989). Fig. III-2 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 hrs after CLP compared to SCLP, and this increase was abolished by treatment with either MPO inhibitor.
We next evaluated the effect of CLP and MPO inhibition on granulocyte macrophage-colony stimulating factor (GM-CSF) expression, a mediator that has been shown to provoke the accumulation of neutrophils in the airways exposed to TNF-α (Laan et al., 2003). Similar to the results for IL-1α and TNF-α, GM-CSF levels increased dramatically after CLP relative to those measured in SCLP (Fig. III-3). This CLP-induced increased was prevented by KYC and ABAH pretreatment, indicating a role for MPO in the elaboration of this proinflammatory mediator. Thus, our data indicates that MPO is essential for the increases in CLP-induced increases in the proinflammatory cytokines IL-1α, TNF-α and GM-CSF in mesentery of septic animals.

Anti-inflammatory cytokines (IL-5 and IL-10) levels in sepsis and effect of pretreatment with MPO inhibitors

While the data listed above reflects the mesentery inflammatory cytokine change for CLP with MPO regulation, the protective anti-inflammatory cytokines can be assessed by measuring the IL-5 and IL-10 levels in sepsis upon the application of MPO inhibitors. In sepsis survivors, there is a trend toward elevated levels of IL-5 compared with non-survivors and has been reported to promote a protective innate immune response during sepsis in an eosinophil-independent manner (Bozza et al., 2007; Linch et al., 2012). Here we report in Fig. III-4 that CLP promotes an increase in plasma IL-5 levels compared to 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.

Treatment with recombinant human IL-10, an anti-inflammatory cytokine has been shown to regulate the progression of sepsis and control the onset of irreversible shock and mortality in CLP-induced sepsis (Berg et al., 1995; Howard et al., 1993; Latifi et al.,
2002; Song et al., 1999; van der Poll et al., 1995). 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. III-5A and B, CLP-induced sepsis was associated with a 2-fold increase plasma levels of IL-10 and a 1.5-fold increase mesentery levels of IL-10 compared to SCLP, whereas, the difference exists between SCLP and CLP in control groups disappear with the application of KYC and ABAH, respectively. These data reaffirm our previous conclusion that MPO involves in IL-10 generation during sepsis.

*Chemokine (MIP-1α, MCP-1, RANTES, LIX, IP-10 and GRO/KC) levels increase in sepsis, changes that are mitigated by concomitant treatment with MPO inhibitors*

CLP induced increases in the expression of the proinflammatory chemokines MIP-1α, MCP-1, RANTES, LIX, IP-10 (Fig. III-6-10). As noted for proinflammatory cytokines, MPO inhibition prevented these increases induced by CLP. In contrast to these data, GRO/KC was not elevated by CLP and MPO inhibitor pretreatment had no effect levels of this chemokine in any group (Fig. III-11). These results indicate that sepsis-induced chemokine expression (but not GRO/KC) is dependent on MPO.

*Expression of the growth factor VEGF is increased by CLP, an effect that is attenuated by MPO inhibition*

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) (Yano et al., 2006). Thus, we wished to determine if expression of this growth factor might also depend on MPO in
septic rats. As shown in Fig. III-12, CLP increased plasma VEGF to 141.48 pg/ml (vs. 95.09 pg/ml for SCLP), a result consistent earlier reports (Yano et al., 2006). 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.

In conclusion, our data support the notion that two MPO inhibitors, KYC and ABAH, effectively prevent CLP-induced release of cytokines (IL-1α, TNF-α, IL-10, IL-5 and GM-CSF), chemokines (MIP-1α, MCP-1, RANTES, LIX and IP-10) and growth factor (VEGF). Taken together with the results presented in Chapter II, these results indicate that MPO plays a key role in the production of inflammation in sepsis.
Figure III-1. MPO inhibition decreases CLP-induced IL-1α in mesentery.

CLP induced marked increases in mesenteric IL-1α, an effect that was attenuated by pretreatment with the MPO inhibitors KYC or ABAH. Means and standard errors of IL-1α (pg/mg protein) for each group were: 2.07 ± 0.29 (SCLP); 26.47 ± 5.55 (CLP); 1.07 ± 0.37 (SCLP + KYC); 11.06 ± 2.94 (CLP + KYC); 2.13 ± 0.52 (SCLP + ABAH, n=8); 9.26 ± 3.25 (CLP + ABAH, n=8). Each set of data represents mean values from six to eight rats. n=6 unless otherwise stated. *, p < 0.05, **, p < 0.01 from ANOVA.
Figure III-2. Increase in mesenteric TNF-α after CLP is reversed by MPO inhibitor treatment.

CLP was associated with a significant increase in mesenteric TNF-α (0.44 ± 0.05, n=6) to the sham surgery (SCLP, 0.27 ± 0.05, n=6). This effect was abolished by pretreatment with the MPO inhibitors KYC (0.19 ± 0.02, n=6) and ABAH (0.16 ± 0.04, n=8). TNF-α levels in the sham CLP groups + MPO inhibitor pretreatment were averaged, SCLP + KYC: 0.12 ± 0.03, n=6; SCLP + ABAH: 0.19 ± 0.06, n=8. *, p < 0.05 and **, p < 0.01.
Figure III-3. MPO inhibition prevents the CLP-induced increase in mesenteric GM-CSF levels.

CLP induced a marked increase of mesenteric GM-CSF compared to that measured in SCLP (no pretreatment bars). This effect was abolished by pretreatment with KYC or ABAH, respectively. Means and standard errors for GM-CSF (pg/mg protein) in each group were: 0.05 ± 0.03 for SCLP, 0 for SCLP + KYC, 0.03 ± 0.03 for SCLP + ABAH; 0.57 ± 0.2 for CLP, not detectable in CLP + KYC or CLP + ABAH. For all the groups n=6, except n=8 in SCLP + ABAH group. * indicates p < 0.05 and ** for p < 0.01.
Figure III-4. KYC and ABAH mitigate CLP-induced increases in plasma IL-5.

CLP increased plasma levels of IL-5 compared to that measured in the SCLP group: 336.42 ± 69.5 in CLP, 241.17 ± 31.29 for SCLP; n=6. Pretreatment with KYC prevented this CLP-induced increase in IL-5 levels: 185.47 ± 19.02 in SCLP + KYC, 167.79 ± 17.03 in CLP + KYC; n=6. A similar response was noted in rats pretreated with ABAH prior to CLP: SCLP + ABAH group, 218.19 ± 26.67; CLP + ABAH group, 220.36 ± 33.07; n=7. * denotes $p < 0.05$, ** $p < 0.01$. 
Figure III-5. KYC and ABAH inhibit CLP-induced increases in IL-10 levels in plasma and mesentery.
(A) IL-10 levels increased plasma obtained from rats subjected to CLP vs SCLP alone (459.02 ± 69.11 for CLP, n=5; 236.21 ± 20.02 for SCLP, n=6). This CLP-induced increase was abolished by pretreatment with KYC (160.06 ± 19.52 for SCLP + KYC group, 237.2 ± 52.86 for CLP + KYC group; n=5) or ABAH (CLP + ABAH group: 423.86 ± 72.96, n=6; SCLP + ABAH group: 385.21 ± 48.46, n=5). (B) A similar pattern of response was noted in mesenteric tissue obtained from animals in these groups (CLP alone vs SCLP alone: 7.11 ± 0.83 and 4.61 ± 0.24, respectively, n=6; SCLP + KYC vs CLP + KYC: 4.53 ± 0.83 and 5.29 ± 0.74, respectively, n=6; SCLP + ABAH vs CLP + ABAH: 4.69 ± 0.92, n=6; and 5.22 ± 1.44, n=7, respectively). *, p < 0.05, **, p < 0.01.
Figure III-6. KYC and ABAH pretreatment attenuate increases in MIP-1α induced by CLP.
(A) MIP-1α in plasma. After the sham and CLP surgery, the plasma MIP-1α in SCLP and CLP groups (n=6) were 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 (n=6 for both groups). Similarly, pretreatment with ABAH reduced MIP-1α concentrations in plasma from both groups: 39.61 ± 5.81 for SCLP + ABAH, 41.4 ± 6.87 for CLP + ABAH; n=8 in each group. (B) MIP-1α in mesentery. A similar pattern of response was demonstrated in mesenteric tissue. Mesenteric MIP-1α levels were increased in samples obtained after CLP compared to sham surgery (SCLP): 28.03 ± 7.79, 10.82 ± 2.09, respectively; n=6 in both groups. ABAH was effective in reducing the increment in MIP-1α levels in mesenteric samples, while KYC was less effective in this regard (mean and standard errors = 0.59 ± 0.08 in SCLP + KYC, and 3.04 ± 0.43 for CLP + KYC, n=6; 0.79 ± 0.22 in SCLP + ABAH, 2.05 ± 0.57 in CLP + ABAH; n=8). * indicates statistical significance, p < 0.05 and **, p < 0.01.
Figure III-7. MPO inhibition limits the increase in plasma and mesenteric MCP-1 induced by CLP.
(A) Comparison of the plasma MCP-1 levels for the SCLP and CLP groups alone and following pretreatment with KYC and ABAH: Plasma MCP-1: 2661.25 ± 144.93 (SCLP), 3856.1 ± 321.46 (CLP), 1695.24 ± 107.88 (SCLP + KYC), 2365.88 ± 245.63 (CLP + KYC, n=5), 2407.69 ± 264.03 (SCLP + ABAH, n=7) and 2955.28 ± 390.35 (CLP + ABAH, n=7). Except for the indicated groups, n=6. (B) Similar comparisons in mesenteric MCP-1: 10.82 ± 2.09 (SCLP), 28.03 ± 7.79 (CLP), 8.75 ± 2.27 (SCLP + KYC), 16.94 ± 3.42 (CLP + KYC), 7.0 ± 1.74 (SCLP + ABAH, n=8) and 9.47 ± 2.41 (CLP + ABAH, n=8). Except for the indicated groups, n=6. *, p < 0.05 and **, p < 0.01.
Figure III-8. Mesenteric RANTES levels are increased by CLP, an effect that is abolished by pretreatment with MPO inhibitors.

Column 1 and 2: Comparison of mesenteric RANTES levels between SCLP (2.94 ± 0.53, n=6) and CLP (4.36 ± 3.51, n=6), showing that this chemokine is increased in septic animals. In SCLP groups, the RANTES concentrations (pg/mg protein) in the presence of KYC and ABAH remained similar to the SCLP without pretreatment in column one: 1.71 ± 0.31 (SCLP + KYC, n=6); 2.43 ± 0.61 (SCLP + ABAH, n=8). The increased RANTES levels induced by CLP was prevented by pretreatment with KYC and ABAH, indicating that MPO is required to produce the increase in mesenteric RANTES in rats subjected to CLP (2.1 ± 0.27 for CLP + KYC, n=6; 2.14 ± 0.86 for CLP + ABAH; n=5). *, p < 0.05, **, p < 0.01.
Figure III-9. Effects of MPO inhibitors to decrease CLP-induced expression of LIX in mesenteric samples.

CLP induced a 4-fold increase of LIX levels in mesenteries versus that measured in rats subjected to SCLP (6.17 ± 1.6 for CLP, 1.64 ± 0.28 for SCLP; n=6). Pretreatment with KYC (SCLP + KYC: 3.17 ± 0.45, n=6; CLP + KYC: 3.89 ± 0.79, n=6) or ABAH (SCLP + ABAH group: 1.51 ± 0.23, n=8; CLP + ABAH group: 2.18 ± 0.54, n=7) attenuated the CLP-induced increases in mesenteric LIX levels. **, p < 0.01.
Figure III-10. Changes in mesenteric IP-10 induced by CLP are attenuated by pretreatment with KYC and ABAH.

While the MPO inhibitors KYC and ABAH do not change the IP-10 concentration in response to SCLP (5.42 ± 0.67 in SCLP, 4.32 ± 0.57 in SCLP + KYC, 4.49 ± 1.24 in SCLP + ABAH; n=6), the increased IP-10 levels noted in mesenteries obtained from rats subjected to CLP surgery are partially decreased by KYC and ABAH (17.63 ± 3.37 in CLP, n=6; 11.87 ± 1.67 in CLP + KYC, n=5; 4.86 ± 3.7 in CLP + ABAH, n=6). *, p < 0.05, **, p < 0.01.
Figure III-11. Plasma GRO/KC levels do not increase following CLP and are unaffected by MPO inhibition.

Although there was tendency for GRO/KC levels to increase in plasma obtained from rats subjected to CLP (no pretreatment bars), this was not statistically significant. Pretreatment with the MPO inhibitors yielded plasma GRO/KC concentrations that were similar in all groups. Means and standard errors for plasma GRO/KC were (in pg/mg protein): CLP alone, $3261.48 \pm 257.12$; SCLP alone, $2505.26 \pm 324.65$; CLP + ABAH, $3145.49 \pm 493.15$, n=7; CLP + KYC, $2492.37 \pm 445.59$, n=4; SCLP + KYC, $2084.62 \pm 282.73$; SCLP + ABAH, $2191.29 \pm 538.17$. n=6 unless otherwise indicated.
Figure III-12. KYC abolishes and ABAH partially reduces CLP-induced increase in plasma VEGF.

Plasma VEGF was increased by CLP (141.48 ± 11.76, n=6) relative to that measured after SCLP (95.09 ± 10.25, n=6). This CLP-induced increase in plasma VEGF was completely prevented by an addition of KYC (49.59 ± 13.2, n=5) and partially mitigated by ABAH (111.18 ± 16.31, n=8). Plasma VEGF averaged as 57.47 ± 11.15 and 77.08 ± 13.69 in the groups subjected to SCLP + KYC (n=6) and SCLP + ABAH (n=6), respectively. * indicates $p < 0.05$, while ** indicates $p < 0.01$. 
v. Discussion

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. Since CLP involves laparotomy, manipulation of the cecum, and placing a ligature to occlude the cecum prior to puncture, a SCLP group was used as a control to ascertain the effect of manipulations associated with these surgical procedures versus that of the infection itself. To perform this expression profile, we first need to ask: what time following surgery is a good starting point for this analysis? With respect to our findings in Chapter II, significant neutrophil activation and the related tissue MPO accumulation (Fig. II-3), increased 2-ClFA (18:0 Cl) (Fig. II-4), increased venular protein leakage (Fig. II-5), acute lung injury (Fig. II-6), enhanced leukocyte and platelet adhesive interactions (Fig II-7-10), mast cell activation and PAI-1 expression (Fig II-11, 12) were noted at 6 hrs. 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 hrs 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, the first 3 being proinflammatory and the last 2 being anti-inflammatory), chemokines (MIP-1α, MCP-1, RANTES, LIX, and IP-10, but not GRO/KC), and the growth factor, VEGF were increased 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) (Rittirsch et al., 2008).

Since distinct cytokine profiles are associated with sepsis severity, organ failure, and mortality (Bozza et al., 2007), current literature offers no consensus opinion as there are multiple studies both in support and against the usefulness of cytokines as prognostic biomarkers (Ventetuolo and Levy, 2008). Regardless of relative lack of sensitivity and specificity for single cytokines as markers of sepsis severity, an overabundant early pro-inflammatory mediators, notably IL-1 and TNF-α, indeed contribute to the unfavorable consequences following sepsis (Bone et al., 1997; Van Zee et al., 1991), and have the ability not only to induce expression of other chemokines and inflammatory mediators, but also induce endothelial cell dysfunction and pro-thrombotic events (Dinarello, 1987; Fong et al., 1989; Gershenwald et al., 1990; Levi et al., 1994; Okusawa et al., 1988; van der Poll et al., 1994). Moreover, Ertel et al. induced sepsis by CLP and observed a persistent elevation of plasma TNF and a steadily increasing IL-1 plasma concentrations compared to the sham group (Ertel et al., 1991).

In the light of recent progress, the classical view that neutrophils function as simple professional killers of invading pathogens (Nathan, 2006) has been expanded by the acknowledgement 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 (Cassatella, 1995; Kasama et al., 2005; Scapini et al., 2000). It is now recognized that neutrophil-derived cytokines contribute to the inflammatory responses in sepsis that can be secreted to an extent that the activation/amplification of host defense is overexuberant, resulting in damage to host
tissue injury (Holdsworth and Gan, 2015). In Chapter II of this dissertation, we showed that CLP-induced sepsis induces significant MPO accumulation in tissues, venular protein leakage, acute lung injury, leukocyte and platelet adhesive interactions, and mast cell activation (Figs. II-3, 5-11), effects that were largely abolished by MPO inhibition. We also showed that CLP causes the formation of proinflammatory 2-ClFA and expression of PAI-1, effects that were also attenuated by pretreatment with MPO inhibitors (Figs. II-4, 5-12). Given the facts that 2-ClFA and PAI-1 are capable of producing the same proinflammatory changes as sepsis, which many cytokines and chemokines are also capable of, 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 provided the evidence that MPO negatively regulates the expression of pro-inflammatory cytokines and chemokines such as MIP-1α, MIP-1β, IL-1α, IL-1β, and TNF-α, by zymosan-activated mouse neutrophils (Endo et al., 2016). 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 pro-adhesive responses between leukocytes, platelets and the endothelium and neutrophil
accumulation in the tissues that may also involve MPO-dependent formation of chlorinated lipids and PAI-1 expression (Figure III-13).

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 pro-inflammatory cytokines exhibiting the largest increases after CLP, IL-1α, TNF-α and GM-CSF, were not elevated in groups treated with the MPO inhibitors (last four columns in Figs. III-1, 2 and 3). 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 (Figs. III-4 and 5). 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.

It is well known that noxious stimuli lead to rapid production of pro-inflammatory cytokines and chemokines, such as Eotaxin, G-CSF, GM-CSF, GRO/KC, MCP-1, MIP-1 and RANTES (Orman et al., 2011), which play important roles in regulating the magnitude and sequence of leukocyte trafficking during inflammatory response (Luster, 1998; Rollins, 1997). 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 (Berkman et al., 1995a; Berkman et al., 1995b; Christman et al., 1992; Kasama et al., 1993; Wolpe et al., 1988). 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 (Yano et al., 2006).

While 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. One possibility is that chlorinated lipids may use the scavenger receptor, CD36, to initiate responses (Nozaki et al., 1995; Zhao et al., 2018).
Figure III-13. Regulation of cytokines by MPO-derived free 2-CIIFA.

Scheme for introducing CLP, cytokine production and the ensuing responses. Brown arrows indicate that on one hand, by inflammatory stimulus, ligand binding via TLRs and DAMP receptors can promote the cytokine production. On the other hand, in light of similar change patterns of 2-CIIFA and cytokine production, we propose that MPO-derived free 2-CIIFA may also participate in CLP-induced cytokine/chemokine generation.
vi. References


CHAPTER IV: CHLORINATED LIPIDS MEDIATE INFLAMMATORY RESPONSES TO SEPSIS BY PAI-1 AND MAST CELLS

The past two decades have witnessed major breakthroughs in unmasking compounds that are generated by MPO-derived reactive chlorinating species (RCS) targeting of plasmalogens on cell membranes in cardiovascular system. However, further improvement in early detection and treatment for sepsis will require insights into the molecular mechanisms whereby chlorinated lipids promote inflammatory responses that can damage host tissue in sepsis. In this study, we examined the role of PAI-1 and mast cell activation in CLP-induced downstream inflammatory responses. Pretreatment with PAI-1 inhibitor PAI-039 prevented CLP induced leukocyte-endothelial cell interactions and mast cell activation, but not platelet rolling and adhesion. On the other hand, pretreatment with the mast cell stabilizer cromolyn sodium prevented adhesive interactions between leukocytes or platelets with venular endothelium. In chapter II of this dissertation, we showed that all of these inflammatory responses to CLP were attenuated by MPO inhibition as well as the increase in PAI-1 induced by sepsis, suggesting that MPO-derived 2-chloro fatty lipids may serve as a mediator. To gain additional insight regarding the role of 2-ClFA as a mechanistic link between the enzymatic activity of MPO and PAI-1- and mast cell-dependent adhesive interactions, superfused mesenteries with 2-ClHDA in the absence and presence of PAI-039 or cromolyn sodium. PAI-039 pretreatment prevented 2-ClHDA induced leukocyte/platelet-endothelium interactions and slightly reduced mast cell activation. Pretreatment with the mast cell stabilizer cromolyn sodium also prevented these responses. Collectively, these data support the concept that CLP-induced, MPO-dependent formation of 2-ClHDA may
activate mast cells and cause release of PAI-1 to promote adhesive interactions between the endothelium and leukocytes and platelets.
ii. Introduction

Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor expressed by vascular endothelial cells, smooth muscle cells, and several other cell types (Ji et al., 2016). Although best-known for its primary function as an inhibitor of fibrinolysis, PAI-1 also regulates cell migration in two ways: (1) by inhibiting pericellular plasmin formation and binding to vitronectin in the extracellular matrix, thereby blocking interactions between vitronectin and $\alpha_{v}\beta_3$ integrin and the plasminogen activator receptor (Holdsworth and Gan, 2015; Walz et al., 1987; Yoshimura et al., 1987); (2) by binding to low-density lipoprotein receptor-related protein 1 (LRP1) and activating a Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway (Waage et al., 1989). By the latter mechanism, Praetner et al. provided evidence indicating that PAI-1 directs neutrophil trafficking during I/R injury by rapid immobilization of PAI-1 on the surface of microvascular endothelial cells and transmigrating leukocytes (Praetner et al., 2018). The immobilized PAI-1 provokes affinity changes in $\beta_2$ integrins expressed on rolling neutrophils, facilitating their firm adhesion to endothelium and subsequent transmigration into tissues, which is associated with endothelial barrier dysfunction. In addition, I/R-induced cytokine formation provokes endothelial cells and other cells to release PAI-1 (Bernat et al., 1995; Fears and Loskutoff, 1997). In conjunction with our data in second chapter indicating that CLP-induced increases in total 2-ClFA and PAI-1 in plasma are attenuated by MPO inhibitors, we hypothesized that sepsis-induced, MPO/HOCl system-derived chlorinated lipids promote PAI-release to mediated inflammatory responses to sepsis. To address this issue, we examined the effect of
tiplaxtinin (PAI-039), a potent and selective PAI-1 inhibitor (Slifka and Whitton, 2000) on leukocyte-/platelet-endothelial interactions and mast cell activation induced by CLP.

Mast cells (MCs) are present in tissues under normal physiologic conditions, residing between parenchymal cells in close proximity to blood vessels, lymphatics, and nerves. This unique perivascular, peri-lymphatic, and perineuronal location, coupled with the vast array of mediators they can release, allows MCs to function as sentinels that can rapidly respond to inflammatory stimuli (Gabay and Kushner, 1999). Due to the fact that mast cell density is very high in gastrointestinal tissues, it comes as no surprise that MCs play a much more prominent role in the inflammatory response to stimuli such as ischemia/reperfusion (I/R) in the small intestine than in other tissues (Bortolotto et al., 2004; Kanwar et al., 1998). In small intestine, mast cells are classified into two categories: (1) connective tissue mast cells in submucosa and muscularis layers in the intestinal wall and mesentery; and (2) mucosal mast cells (Miller and Pemberton, 2002). Mast cells have been implicated in the regulation of vasomotor tone, endothelial cell activation, microvascular permeability and edema formation, angiogenesis, and leukocyte migration (Klein et al., 1989; Malaviya et al., 1996; Yano et al., 1989; Zhang et al., 1992). Mast cells release proteases upon activation. Since these proteases are stored in exceptionally high levels and are present as fully active enzymes, they can immediately produce tissue injury once released (Pejler et al., 2010; Schwartz et al., 1987; Schwartz et al., 1981) owing to their hydrolytic activity towards cleavable substrates. In addition to releasing a host of proinflammatory mediators, connective tissue mast cells contain chymase, an enzyme that appears to be responsible for most of the angiotensin II formed at sites of tissue injury. This is important because angiotensin II plays an important role
in increasing microvascular permeability by severing adhesion proteins comprising junctional complexes between endothelial cells (Ebihara et al., 2005; Granger and Granger, 2011; Gu et al., 2009; Kubes and Granger, 1996; Lawrence et al., 2004; Scudamore et al., 1998) and alters the bioavailability of the anti-adhesive molecule nitric oxide secondary to its action to activate NADPH oxidase during inflammatory states, to promote leukocyte-endothelial cell interactions (Yusof et al., 2007). Moreover, during reperfusion tissue resident mast cells are activated (Kanwar et al., 1998; Kubes and Granger, 1996), secreting mediators that promote adhesion molecule expression and thereby contributing to post-ischemic leukocyte infiltration. Therefore, considering the fact that CLP elicits mast cell activation, an effect that was prevented by concomitant treatment with MPO inhibitors, we hypothesized that sepsis-induced MPO/HOCl system-derived chlorinated lipids may contribute to mast cell activation, which in turn promote adhesive interactions between leukocytes/platelets with the endothelium.

In a final series of experiments, we evaluate the effects of PAI-1 inhibition and mast cell stabilization on the effects of chlorinated lipids, in lieu of CLP, to promote proinflammatory responses in the microcirculation. Results of these experiments mimicked those we observed in response to CLP, suggesting the possibility that chlorinated lipids generated by an MPO-dependent mechanism serves as mediator of downstream inflammatory responses.
iii. Materials and methods

Study PAI-039 and cromolyn sodium in sepsis

Animals and sepsis model
See materials and methods in Chapter II.

PAI-039 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 (i.p.) 1 hr prior to surgery for CLP and SCLP groups, then the second dose was offered 1 hr before data collection (Ji et al., 2016).

Cromolyn sodium pretreatment
Cromolyn sodium (50 mg/kg) was applied (i.p.) 30 min before surgery and the second dose was at 30 min before the measurements (Kiraly et al., 2000).

Chemicals and solution compositions
PAI-039 powder was provided by Dr. William P. Fay at University of Missouri and kept at -20°C. PAI-039 was dissolved in DMSO (22 mg/ml), diluted in 1 x PBS (10 µl/5 ml), stored as a 44 µg/ml stock at -20°C, and kept it fresh that usually applied within two weeks (Ji et al., 2016).

Cromolyn sodium salt was purchased from Sigma-Aldrich and stored as required. Dissolved this powder in saline and stocked as a 50 mg/ml stock at -20°C and kept it fresh that usually applied within two weeks (Jiang et al., 2018).

Intravital Microscopy
See materials and methods in Chapter II.

Leukocyte-/platelet-endothelium interactions

See materials and methods in Chapter II.

Mast cell activation

See materials and methods in Chapter II.

Study chlorinated lipids and non-chlorinated lipids superfusion

See methods in APPENDIX I: Methods.

Study PAI-039 in rats with 2-ClHDA superfusion

General procedure

2-ClHDA was kindly provided by Dr. David A. Ford from Center for Cardiovascular Research, Saint Louis University. Experimental group rats were pretreated with PAI-039 (44 µg/kg, i.p.) 1 hour prior to the initiation of 2-ClHDA superfusion. Anesthetized rats as previously described, then exteriorized the mesentery and superfused with 2-ClHDA (10 µM) for 90 minutes. On the other hand, without any pretreatment, superfused mesentery with HDA and 2-ClHDA at the same molar concentration as negative and positive control. Evaluated inflammatory responses, thrombogenic effects, and mast cell activation at baseline, 30 min, 60 min, and 90 min upon superfusion.

Data analysis and statistics

MetaMorph (Nashville, TN) was used to estimate leukocyte/platelet-endothelium interaction and mast cell activation. All data were shown as sample mean ± standard error of the mean; n represents the number of rats in each set of experiment. Multiple
comparisons were initially evaluated by two-way ANOVA and repeated measures ANOVA accordingly, then followed by Fisher’s LSD test. Graphpad Prism 6.0 was used to generate all statistical values. A $p < 0.05$ is the level of statistical significance, and $p < 0.01$ indicates highly significant.
iv. Results

Roles of PAI-1 and mast cells in CLP-induced inflammatory responses

In Chapter II of this dissertation, we showed that CLP was associated with increased free 2-CIFA (18:0 Cl) in circulation (Fig. II-4), total PAI-1 in plasma (Fig. II-12) and mast cell activation in interstitial space (Fig. II-11), effects that were attenuated by MPO inhibition. The aims of this study were to determine if CLP-induced leukocyte and platelet rolling and firm adhesion were dependent on PAI-1 and mast cell activation. To address the role of PAI-1, we treated septic rats with a selective PAI-1 inhibitor, PAI-039. In a second series of experiments, we examined the question of whether treatment with a mast cell stabilizer would limit inflammatory responses to CLP. We undertook the latter study for two reasons: First, activated human mast cells release a striking amount of functionally active PAI-1 when activated, which reasonably implies an indirect role for these sentinel cells in the development of sepsis (Luster, 1998). Second, mast cells have been shown to aggravate sepsis by functional crosstalk between peritoneal mast cells and macrophages involving the immediate release of pre-stored IL-4 by mast cells (Luster, 1998). We thus speculated that treatment with the mast cell stabilizer, cromolyn sodium, may limit inflammatory responses in sepsis.

To test these postulates, we used the same CLP model employed in earlier chapters in this dissertation. Fig. IV-1 shows the effects of PAI-039 and cromolyn sodium on the ability of CLP to increase the numbers of rolling leukocytes. Both inhibitors attenuated leukocyte rolling induced by sepsis. Taken together, our data suggests that both PAI-1 and activated mast cells act to promote leukocyte rolling in CLP-induced sepsis. The data
presented in Fig. IV-2 indicates that CLP-induced stationary leukocyte adhesion also occurs by PAI-1- and mast cell-dependent mechanisms.

Sepsis can induce a prothrombogenic phenotype characterized by platelet activation and hyper-aggregation and development of disseminated intravascular coagulation (Becchi et al., 2006; Guclu et al., 2013). In view of this, we postulated that pretreating with PAI-039 or cromolyn sodium may effectively limit platelet interactions with endothelial cells. Fig. IV-3 shows a summary data regarding the effect of CLP to increase platelet rolling in mesenteric microcirculation relative to that measured in rats subjected to SCLP. Surprisingly, PAI-039 failed to prevent the increase in platelet rolling invoked by CLP. On the other hand, treatment with the mast cell stabilizer cromolyn sodium, reduced platelet rolling to levels not different from that noted in rats subjected to SCLP alone. A similar pattern of response was noted regarding platelet adhesion, with CLP inducing a marked increase in the number of firmly adherent platelets (Figure IV-3). Pretreatment with PAI-039 failed to block this response while cromolyn sodium completely prevented CLP-induced platelet adhesion. These results suggest that activated mast cells play a much more prominent role in sepsis-induced platelet rolling and adhesion, with both adhesive processes appearing independent of PAI-1.

_Chlorinated lipid mediates inflammatory responses that mimic those invoked by sepsis_

We have presented limited correlative evidence suggesting that free 2-ClFA (18:0 Cl) may serve as a downstream mediator of MPO-instigated inflammatory responses in CLP. As discussed earlier, compounds acting to scavenge 2-ClFA as they are produced are not currently available. In addition, cellular receptors for this chlorinated lipid have yet to be identified. Consequently, direct evaluation of the role for 2-ClFA is not yet possible.
previous work (Yu et al 2019), we took the alternative strategy of correlating the inflammatory responses elicited by direct application of chlorinated lipids (2-ClHDA and 2-ClPA) and corresponding control non-chlorinated lipids (HDA and PA) to the mesentery to those instigated by CLP, as described below and in Appendix I. In Chapter II of this dissertation, we established that CLP induces an increase in 2-ClFA, an effect that was attenuated by the MPO inhibitors KYC and ABAH. Since CLP-induced neutrophil infiltration, venular protein leakage, acute lung injury, leukocyte and platelet adhesive interactions, and mast cell activation were also limited by concomitant MPO inhibition, we postulated that 2-ClFA may serve as a trigger for the development of these responses.

Establishment of 2-ClHDA/PAI-1 axis to sepsis

Using the data presented in Appendix I, we conducted new studies directed at examining the effect of PAI-039 on the effects of exogenous 2-ClFA to produce inflammatory responses in lieu of CLP. In the MPO/HOCl/2-ClHDA system, chlorinated lipid products such as 2-ClHDA and other types of chlorinated aldehyde, are formed in response to reactive chlorinating species (RCS) attacking membrane plasmalogens (Adam et al., 2014; Albert et al., 2001; Anbukumar et al., 2010; Malle et al., 2000; Thukkani et al., 2005; Ullen et al., 2013), leading to the formation of 2-ClPA, a type of 2-ClFA. In our experiments, free 2-ClFA and total PAI-1 in circulation indeed increase following CLP (Figs. II-4 and II-12), whereas inhibiting PAI-1 with PAI-039 prevents the reactions for CLP (Figs. IV-1 to 5). Therefore, we next tested the effect of PAI-039 on 2-ClHDA-mediated inflammatory responses. Fig. IV-6 shows that the effect of 2-ClHDA superfusion to increase leukocyte rolling is abolished by concomitant treatment with PAI-
Similar results were obtained regarding leukocyte adhesion (Fig. IV-7) and platelet rolling and adhesion (Figs. IV-8 and 9). These observations indicate that 2-ClHDA induces increase in leukocyte and platelet adhesive responses that mimic the responses to CLP. Importantly, these proinflammatory responses to exogenous 2-ClHDA are prevented by PAI-1 inhibition, which again mimics the responses to PAI-039 in CLP.

The data in Fig. IV-10 illustrates the effects of mesenteric superfusion 2-ClHDA on mast cell activation in the absence and presence of PAI-039 in the 2-ClHDA superfusion. While superfusion with non-chlorinated lipid was without effect, 2-ClHDA produced an increase in mast cell activation, an effect which was attenuated by PAI-039. These data indicate that PAI-1 is partially responsible for mast cell activation instigated by chlorinated lipid superfusion.

Taken together, this new data indicates that PAI-1 plays an important role as a downstream mediator of leukocyte and platelet rolling and adhesion as well as mast cell activation that occurs in response to exogenous 2-ClHDA superfusion. These responses mimic those noted with CLP, providing additional correlative support for the concept that chlorinated lipids may serve as a mechanistic link between CLP-induced MPO activity and downstream inflammatory responses manifested in the microcirculation of septic animals. More definitive analysis of this question will require development of chlorinated lipid scavengers or identification of receptors that bind 2-CIFA. Once identified, use of receptor blockers for these putative 2-CIFA receptors will be required to more firmly establish cause and effect relationships.
Figure IV-1. Effects of PAI-039 and cromolyn sodium on CLP-induced leukocyte rolling.

As shown in first pair of columns, compared to SCLP (1006.34 ± 24.19, n=6), CLP remarkably increases the number of rolling leukocytes (2404.42 ± 91.40, n=6). This CLP-induced increase in leukocyte rolling was attenuated by pretreating rats with PAI-1 inhibitor, PAI-039 (1516.33 ± 57.91, n=6) or the mast cell stabilizer, cromolyn sodium (1517.34 ± 96.88, n=6). Values in SCLP + PAI-039, and SCLP + cromolyn sodium groups are as follows: 1519.54 ± 25.77, n=6; 1570.36 ± 34.20, n=5. ** indicates statistical significance at $p < 0.01$. 
Figure IV-2. PAI-039 and cromolyn sodium inhibit increased leukocyte adhesion induced by CLP.

The number of firmly adherent leukocytes increased after CLP (260.79 ± 40.1, n=6) relative to SCLP (143.42 ± 30.37, n=6). This CLP-induced increase in leukocyte adhesion was largely abolished by pretreatment with PAI-039 (193.01 ± 33.07, n=6) or cromolyn sodium (118.59 ± 13.30, n=6). Neither PAI-039 nor cromolyn sodium affected leukocyte adhesion in rats subjected to SCLP (175.35 ± 21.73, n=6 and 159.48 ± 31.16, n=5, respectively). ** indicates values statistically different from CLP alone at $p < 0.01$. 

![Graph showing leukocyte adhesion](image-url)
Figure IV-3. Disparate effects of PAI-1 inhibition vs mast cell stabilization on platelet rolling induced by CLP.

CLP increases the number of rolling platelets ($727.46 \pm 159.71$) relative to SCLP ($331.1 \pm 99.66$). Treatment with PAI-039 failed to reduce leukocyte rolling ($782.02 \pm 130.29$), while cromolyn sodium was quite effective in this regard ($447.67 \pm 142.98$). PAI-039 exerted little effect on platelet rolling in SCLP ($395.88 \pm 122.18$) while there was a non-statistical increase in platelet rolling in SCLP rats pretreated with cromolyn sodium ($646.95 \pm 173.21, n=5$). Except where specifically indicated, $n=6$. ** indicates values statistically different from CLP alone at $p < 0.01$. 
Figure IV-4. Modulation of CLP-induced platelet adhesion by cromolyn sodium but not PAI-039.

The leftmost pair of data bars presented above indicates that CLP increases the number of firmly adherent platelets ($79.76 \pm 14.9$, $n=6$) compared to SCLP group ($26.97 \pm 12.45$, $n=6$). The results depicted in the middle bars indicate that pretreatment with PAI-039 was without effect on platelet adhesion in rats subjected to SCLP ($35.33 \pm 18.5$, $n=6$) or CLP challenge ($67.21 \pm 18.47$, $n=6$) relative to SCLP or CLP alone. On the other hand, cromolyn sodium pretreatment reduced the number of firmly adherent platelets induced by CLP ($13.69 \pm 5.44$, $n=6$). Interestingly, mast cell stabilizer treatment appeared to augment platelet adhesion in the SCLP group ($56.27 \pm 12.89$, $n=5$) when compared to SCLP alone. * indicates statistical significance at $p < 0.05$, and ** indicates $p < 0.01$. 
Figure IV-5. Mast cell activation induced by CLP is abrogated by pretreatment with PAI-039 and cromolyn sodium.

As shown in the leftmost pair of bars, CLP induces a marked increase in the number of activated mast cells. This increase in CLP-induced mast cell activation was abolished by pretreatment with PAI-039 and cromolyn sodium. These agents were without effect in rats subjected to SCLP. Quantification as follows: 21.32 ± 1.31 for CLP, 5 ± 0.48 for CLP + PAI-039, and 4.3 ± 0.81 for CLP + cromolyn; 3.72 ± 1.28 for SCLP, 6.35 ± 0.46 for SCLP + PAI-039, and 5.82 ± 0.99 for SCLP + cromolyn. n=6 for all groups except for the SCLP + cromolyn group, where n=5. **, p < 0.01.
Figure IV-6. Effects of PAI-039 on chlorinated lipid-induced leukocyte rolling.

The number of leukocytes rolling (per mm$^2$ per minute) in mesenteric microcirculation superfused with exogenous HDA (negative control), 2-ClHDA (positive control), and 2-ClHDA superfusion with PAI-039 pretreatment (experimental group). As shown in the first two clusters, the chlorinated lipid (2-ClHDA) promoted leukocyte rolling, whereas the non-chlorinated lipid (HDA) failed to exert an effect. Specifically, at 30min, 60 min and 90 min superfusion, as against to baseline, the group superfused with 2-ClHDA showed a significant increase in leukocyte rolling that remained stable once elevated by $4.30 \pm 0.95$, $4.07 \pm 0.70$, $4.64 \pm 0.79$-fold; whereas, compared with its baseline, the mean fold change of leukocyte rolling in HDA group rises very slightly at the matching time point ($1.54 \pm 0.76$, $1.47 \pm 0.41$, $1.96 \pm 0.75$-fold). On the contrary, PAI-039 pretreatment prevented the 2-ClHDA induced increase ($0.92 \pm 0.06$, $1.51 \pm 0.07$, and $1.13 \pm 0.04$-fold). n=6. **, $p < 0.01$. 
Mean fold changes in leukocyte adhesion in the same three groups were also evaluated. It is interesting to note that, there were minor increases of mean fold change of leukocyte adhesion in both HDA and 2-ClHDA groups along with superfusion; However, when compared to their corresponding baselines, no significant differences were determined: 2.39 ± 2.62, 2.22 ± 2.55, 2.24 ± 1.04-fold increase for HDA group, and 4.73 ± 3.08, 2.96 ± 1.76, 1.56 ± 0.7-fold increase for 2-ClHDA group. However, as postulated, even the slight increase was brought back to a level close to the mean fold change at 0 min, achieved by the additional treatment with PAI-039 prior to 2-ClHDA challenge, as follows: 1.30 ± 0.16 at 30 min; 1.28 ± 0.11 at 60 min; 1.30 ± 0.20 at 90 min. n=6.
Figure IV-8. PAI-039 prevents platelet rolling elicited by exogenous 2-CIHDA.

2-CIHDA superfusion significantly increased platelet rolling, averaging 2.37 ± 0.72, 2.20 ± 0.59, 2.16 ± 0.82-fold increase over baseline after 30, 60, and 90 min, respectively. HDA superfusion was without effect over the same superfusion time frame, averaging 0.69 ± 0.37, 0.57 ± 0.21, and 0.58 ± 0.29-fold change versus the baseline. As hypothesized, the increased platelet rolling resulting from 2-CIHDA challenge was largely abolished by pretreating with PAI-039, averaging 0.45 ± 0.26, 0.34 ± 0.20, 0.26 ± 0.17-fold change over the same time frame, indicating that 2-CIHDA-induced platelet rolling is PAI-1 related. n=6. * indicates p < 0.05.
Figure IV-9. Effects of PAI-039 on platelet adhesion induced by 2-ClHDA superfusion.

The platelet adhesion from the rat mesenteric venules was quantitated, normalized to baseline and graphed against time. The number of adhesive platelets was very low or even undetectable before lipid treatment in both groups, and this situation persisted throughout the observation period in mesenteries superfused with HDA (1.35 ± 0.50, 1.44 ± 0.64, and 1.43 ± 0.65-fold increase at 30, 60, and 90 min superfusion, compared to baseline). On the other hand, superfusion with 2-ClHDA promoted significant increases in platelet adhesion to the venular walls (2.74 ± 0.39, 2.75 ± 0.43, 3.73 ± 0.59-fold increase after 30, 60, and 90 min superfusion, respectively, as against to its baseline). This response to 2-ClHDA superfusion was abolished by treatment with the PAI-1 inhibitor (PAI-039), averaging 0.39 ± 0.39, 0.28 ± 0.18, and 0.22 ± 0.14-fold change after
30, 60 and 90 min respectively. Each mean fold change in the bar graph represents mean values from five to eight rats. ** indicates $p < 0.01$. 
Mast cell activation was evaluated in perivascular interstitium for three groups at the end time point of experiment. Quantitative analysis revealed that 2-ClHDA (7.71 ± 1.10 averaged activated mast cells) provoked enhancement in mast cell activation when compared to HDA superfusion (4.05 ± 0.28). The effect of 2-ClHDA to increase mast cell activation was slightly but significantly reduced (6.85 ± 0.72) by application of PAI-039 prior to the initiation of 2-ClHDA superfusion. n=6. *, p < 0.05, **, p < 0.01.
v. Discussion

PAI-1 and mast cells in CLP-induced sepsis

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 2-ClHDA as a downstream mediator. 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 post-ischemic tissues (Anbukumar et al., 2010; Thukkani et al., 2005). We provided evidence that CLP-induced sepsis is associated with proinflammatory responses that are prevented by treatment with a PAI-1 inhibitor (PAI-039) or mast cell stabilizer (cromolyn sodium). In view of the exacerbated 2-ClFA generation in sepsis, our studies regarding the contributions of PAI-1 and mast cell activation to enhanced leukocyte and platelet adhesive interactions induced by exogenous chlorinated lipids provides new correlative evidence for a potential role for 2-ClHDA as a potential mediator for pathophysiological responses to sepsis.

How PAI-1 contributes to sepsis-induced adhesive interactions is unclear. Although PAI-1 has well-known antifibrinolytic properties (Declerck and Gils, 2013; Loskutoff et al., 1983), a number of non-fibrinolytic functions have been described in recent years (Kozlova et al., 2015; Praetner et al., 2018). Recent studies indicate that PAI-1 promotes neutrophil infiltration and tissue injury induced by ischemia/reperfusion. This effect is mediated through low-density lipoprotein receptor-related proteins (LRP-1)-dependent signaling pathway (Kozlova et al., 2015; Praetner et al., 2018). This coupling between PAI-1 and cell migration in I/R is consistent with other work showing that PAI-1
regulated smooth muscle cell migration by competitively blocking cell binding interactions with extracellular matrix vitronectin (Stefansson and Lawrence, 1996).

About a decade ago, by studying binding events of PAI-1, which does not possess its own cellular receptor, Dupont et al., proposed that it can be bound to cell surface, internalized in a complex with urokinase-type plasminogen activator (uPA), and the uPA receptor through association with the scavenger receptor LRP-1 (Dupont et al., 2009). At around the same time, it was discovered that LRP-1 can interact with β2-integrins on leukocytes (Spijkers et al., 2005). Specifically, LRP appears to regulate α1β2-integrin clustering, as such, β2-integrins-mediated adhesion to endothelial cells, supporting a previously unrecognized link between LRP and the inflammatory system. Although the proposition that PAI-1 modifies cell migration by binding to LRP-1 in sepsis awaits more studies to substantiate, this hypothesis is consistent with the previous report on the possible signaling pathway including a LRP-1-dependent manner via β-catenin and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) in the effect of PAI-1 to modulate cell migration (Kozlova et al., 2015).

Possible interaction between mast cells and PAI-1

The results of the present study support an important role for PAI-1 in sepsis-induced mast cell activation. Since stabilizing mast cells with cromolyn sodium was as effective as PAI-1 inhibition with PAI-039 in preventing CLP-induced leukocyte rolling and adhesion (Figs. IV-1, -2 and -5), our work suggests that CLP-induced mast cell activation may involve MPO-dependent PAI-1 generation. On the other hand, PAI-1 did not appear to play a role in CLP-induced platelet rolling and adhesion (Fig. IV-3 and 4).
It is well known that mast cells release a large number of distinct biologically active mediators, such as IL-1, TNF-α, histamine, angiotensin II, etc., that promote leukocyte-endothelial adhesive interactions (Serafin and Austen, 1987; Zhang et al., 1992). Of note, two decades ago, Christian et al., presented evidence indicating primary human lung mast cells released enzymatically active tissue plasminogen activator (tPA) but did not express detectable PAIs (Sillaber et al., 1999). At about the same time, Cho et al showed that human mast cells secreted abundant amounts of functionally active PAI-1 upon the increased synthesis of PAI-1 mRNA, in a manner that is different from the secretion of tPA (Cho et al., 2000). More recently, mast cell-derived exosomes were shown to stimulate endothelial cells to secrete PAI-1, detectable at the levels of PAI-1 promoter activity, PAI-1 mRNA and protein synthesis (Al-Nedawi et al., 2005). These studies suggest that mast cells may promote increases in tissue PAI-1 to mediate leukocyte rolling and adhesion in sepsis. In future studies, we propose to determine whether the increases plasma total PAI-1 induced by sepsis we reported in Chapter II of this dissertation will be prevented by treatment with mast cell stabilizers to more firmly establish this link.

Chlorinated lipids-induced mast cell activation and other inflammatory responses in sepsis.

Since neutrophil infiltration appears to be essential for parenchymal cell injury in sepsis, identifying the mechanisms responsible for these inflammatory responses is critical to understand the pathology of damage to the host after bacterial invasion. More than a decade ago, it is shown that chlorinated fatty aldehydes can be formed by the MPO/HOCl/RCS system targeting of membrane plasmalogens (Albert et al., 2001;
Thukkani et al., 2002). A recent report also documented that chlorinated fatty aldehyde is metabolized to 2-chloro-fatty acids and 2-chloro-fatty alcohols (Anbukumar et al., 2010; Wildsmith et al., 2006). It was striking to note that chlorinated lipids have been shown to accumulate in not only activated neutrophils and monocytes, but also in infarcted myocardium and human atherosclerotic lesions (Thukkani et al., 2002; Thukkani et al., 2005; Thukkani et al., 2003). In addition, 2-chlorofatty aldehyde is a potent chemoattractant and can elicit the surface expression of P-selectin in endothelial cells (Thukkani et al., 2002; Thukkani et al., 2003). In addition, they inhibit nitric oxide (NO) production, an important anti-adhesive molecule (Marsche et al., 2004). In an earlier study, we demonstrated effects from two types of chlorinated lipids (2-ClHDA and its derivative, 2-ClPA) induce significant inflammatory effects to an extent that are much greater than non-chlorinated controls (HDA and PA, respectively).

2-ClHDA/PAI-1 axis

While our studies implicate a role for MPO-dependent PAI-1 production in the increased leukocyte-endothelial cell interactions and mast cell activation, it was not clear whether MPO-derived chlorinated lipids played a role as a downstream mediator to upregulate PAI-1. Since specific scavengers for 2-ClFA are not currently available and the receptor(s) for 2ClFA is unknown, we adopted a correlative analysis approach to gain some insight into the question of whether this chlorinated lipid increased leukocyte-endothelial cell by a PAI-1-dependent mechanism as a potential link in our sepsis scheme (Fig. IV-11).
Figure IV-11. Chlorinated lipids mediate inflammatory response to sepsis by PAI-1- and mast cell-dependent mechanisms.

Brown arrows indicate that on one hand, both CLP and chlorinated lipids elicit inflammatory responses by PAI-1 and mast cell activation; On the other hand, in light of MPO-derived free 2-CIFA in CLP-induced sepsis, we thus propose that chlorinated lipids mediate inflammatory response to sepsis by PAI-1 and mast cell activation.
vi. References

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CHAPTER V: SUMMARY AND FUTURE DIRECTIONS

In chapter II and III, I presented evidence implicating a role for MPO in CLP-induced neutrophil infiltration, venular albumin leakage, acute lung injury, adhesive interactions between leukocytes and platelets with venular endothelium, mast cell activation, and production of proinflammatory mediators (2-ClFA, PAI-1, the cytokines IL-1α, TNF-α, and GM-CSF, and the chemokines MIP-1α, MCP-1, RANTES, LIX, and IP-10), the growth factor VEGF, and anti-inflammatory cytokines (IL-5 and IL-10). In these studies, two structurally dissimilar and mechanistically distinct MPO inhibitors (KYC and ABAH) were used to rule down the possibility of off-target effects. In most instances, the proinflammatory responses induced by CLP were prevented by both inhibitors. Notable exceptions were the sepsis-induced increases in platelet rolling, which were abolished by KYC, but not ABAH, pretreatment. It is possible that higher doses of ABAH may have been required to limit CLP-induced increases in 2-ClFA and platelet rolling, but this was not possible because preliminary studies demonstrated that the higher volume of ABAH diluent (DMSO) in the absence of ABAH (i.e., vehicle control experiments) was as effective as KYC in preventing these proinflammatory responses to sepsis. This is likely since DMSO is a powerful antioxidant that may detoxify reactive oxygen species that are required to fuel MPO-dependent mediator production. To address this potential data interpretation issue, we propose future studies using HOCl scavengers, as this powerful oxidant is the major reactive species directly formed by MPO. It is also possible that potential off-target effects of KYC that are not shared with ABAH account for its actions.
to prevent platelet rolling. Hydrophobicity is another important characteristic of MPO inhibitors that has impact on in vivo effectiveness. ABAH is rapidly taken up by LDL particles because it is highly soluble in lipids. On the other hand, lysine residues in the KYC molecule increases the hydrophilic properties of this MPO inhibitor, enhancing KYC’s retention in hydrophilic environment in vivo (i.e., blood stream).

Based on inhibitor studies presented in the literature and in Chapter IV of this dissertation, it is likely that the microvascular barrier disruption, acute lung injury and enhanced interactions of leukocytes and platelets with endothelial cells occur secondary to MPO-dependent formation of the proinflammatory mediators listed above. Additionally, CLP-driven, MPO-dependent venular protein leakage induced by proinflammatory mediators may be augmented by the endothelial barrier disrupting effects of VEGF. Since mast cell activation is associated with release of many mediators that promote protein leakage and increased leukocyte/endothelial interactions, mediators released from these sentinel cells likely play an important role in augmenting MPO-dependent proinflammatory effects of CLP.

It should be noted that mast cell activation may represent a double-edged sword to the host. On the one hand, systemic mast cell activation increases mortality during CLP-induced sepsis by overzealous cytokine production causing deleterious inflammatory responses resulting in multiple organ failure (Seeley et al., 2011). Indeed, most of these functions of mast cell are due to production and release of distinct biologically active mediators, including histamine, protease and cytokines (TNF-α) (Plaut et al., 1989). Despite the traditional view of mediators released, a wealth of recent studies provides compelling evidence indicating that activated human mast cells release a striking amount
of functionally active PAI-1, and mast-cell derived exosomes significantly upregulate PAI-1 secretion from endothelial cells (Al-Nedawi et al., 2005; Cho et al., 2000). In the past few decades, it has become clear that mast cell activation induces the expression of endothelial cell adhesion molecules that promote leukocyte infiltration and microvascular barrier disruption in tissues subjected to ischemia/reperfusion challenge (Kalogeris et al., 2012; Kanwar et al., 1998; Kubes and Granger, 1996).

Although not a goal of this dissertation work, it would be very interesting to determine the effect of treatment with mast cell stabilizers on CLP-induced generation of cytokines, chemokines and VEGF. Likewise, it would be most informative to conduct future studies directed at determining the effect of exogenous 2-ClFA, in lieu of CLP, on the generation of cytokines, chemokines and VEGF.

The studies outlined in Chapter IV sought to evaluate the roles of PAI-1 and mast cells in MPO-dependent proinflammatory responses. Using a pharmacologic approach, we demonstrated roles for MPO-dependent PAI-1 and mast cell activation in the effects of CLP to increase leukocyte-endothelial cell adhesive interactions and mast cell activation. However, PAI-1 inhibitor treatment failed to abrogate platelet rolling or adhesion induced by CLP. Thus, it appears that PAI-1 does not play a role in the prothrombogenic effects in this model of sepsis. Based on these observations, we expected that when naïve (no CLP) mesenteries were superfused with exogenous 2-ClHDA, this would induce increases in leukocyte and platelet rolling and adhesion, but that only leukocyte rolling, and adhesion would be prevented by PAI-1 inhibition. However, PAI-039 treatment very effectively abolished platelet adhesive responses invoked by 2-ClHDA. One potential explanation for these apparently disparate findings is that other MPO-dependent
mediators (e.g., cytokines and chemokines) were responsible for platelet adhesive interactions in rats subjected to CLP or that other chlorinated species in addition to 2-ClHDA participated in our septic model. This could be investigated by use of HOCl scavengers, as outlined above.

While our results strongly suggest that MPO-dependent generation of PAI-1 and mast cell activation are critical mediators of the proinflammatory effects of CLP, these studies relied on single pharmacologic reagents to either inhibit PAI-1 or prevent mast cell activation. Although we confirmed that CLP-induced mast cell activation was prevented by cromolyn sodium, it is possible that this agent, as well as PAI-039, exert off-target effects. In future studies, we propose to use other approaches to eliminate PAI-1 signaling or mast cell activation to bolster our conclusions.

PAI-1 levels were elevated in CLP rats, an effect that abolished by MPO inhibition. Moreover, the prominent leukocyte-endothelial interactions induced by CLP or 2-ClHDA superfusion were completely prevented by treatment with the PAI-1 inhibitor, PAI-039 (Chapter IV). While these observations provide strong support for a role for MPO-dependent, PAI-1 mediated leukocyte-endothelial cell interactions in CLP, the downstream mechanisms responsible for these responses are unknown. However, PAI-1 has been shown to bind to cell surface glycosaminoglycans to enhance its biological activity that, consequently, promotes neutrophil adherence to the microvascular endothelium and the subsequent transmigration to the interstitial tissue after ischemia/reperfusion (Gebbink et al., 1993; Urano et al., 1994). PAI-1 interacts with LRP-1, which serves as a multifunctional scavenger and signaling receptor in various biological processes (Gonias and Campana, 2014; May, 2013; Strickland et al., 2014),
including leukocyte trafficking (Weckbach et al., 2014). It is fairly well-established that LRP-1 is expressed on neutrophils. Thus, we propose that immobilized PAI-1 mediates leukocyte/endothelial interactions in the process to sepsis via this neutrophil receptor protein, LRP-1. A possible mechanism to this issue would be PAI-1 accumulated on microvascular endothelial cells is encountered by rolling neutrophils, which triggers affinity changes in β2 integrins, by a process that requires LRP-1, as has been shown to occur after I/R (Praetner et al., 2018). Binding interactions between activated β2 integrin and above-mentioned cell adhesion molecules expressed on endothelial cells, including intercellular adhesion molecule-1 (ICAM-1), leads to firm neutrophil adhesion.

The weakest link in our proposed scheme to explain the mechanistic links between CLP-induced MPO activity and downstream proinflammatory responses exemplified by increased cytokine/chemokine/VEGF/PAI-1 levels, leukocyte and platelet adhesive interactions, mast cell activation, and acute lung injury relates to our proposal that MPO-dependent production of chlorinated lipids serves as the major mediator of these subsequent events. To begin to address this linkage, we showed that 2-ClHDA levels are elevated by CLP and showed that MPO inhibition prevented this response. Moreover, superfusion naïve (no CLP) mesenteries with exogenous 2-ClHDA at concentrations relevant to those measured in sepsis, produced proinflammatory responses that mimicked those evoked by CLP. However, it is possible that HOCl produced by MPO could directly cause these changes or could act to chlorinate biologically important molecules other than lipids to provoke inflammatory responses. We must await development of compounds that specifically and effectively scavenge chlorinated lipids or discover the receptor (and specific inhibitors for that receptor) for chlorinated lipids that may be
responsible for directing their proinflammatory actions to provide more definitive evidence for this concept.

It is important to recognize that while our studies provide important mechanistic insight into the pathologic mechanisms underlying overexuberant inflammatory responses that may provoke host injury in sepsis, strategies will have to be developed that limit MPO-dependent injury to parenchymal cells but allow the immune system to destroy invading bacteria. This is the major challenge to develop therapies that will reduce the morbidity and mortality in sepsis. Thus, one of the most valuable aspects of our research as it currently stands is that our results suggest several new potential biomarkers that could be used to assess the progression and severity of the septic state.

Recently, it has been demonstrated that extravasated neutrophils can re-cross endothelial junctions in an abluminal to luminal direction in a process termed reverse trans-endothelial migration (rTEM) of neutrophils from tissue back to circulation (Jin et al., 2019; Woodfin et al., 2011). These cells can be carried by the circulation to distant sites to provoke multiple organ failure in sepsis. Here I will discuss some ideas for future experiments that aim to address this question: how do activated neutrophils from circulation disseminate?

We may use flow cytometry as tools to study the dissemination that assume different phenotypes of TEM and rTEM neutrophils. For examples, it has been proposed that the rTEM neutrophils constitute a phenotypically and functionally differentiated population, with a characteristic phenotype (CD54^{high}, CXCR1^{low}) (Buckley et al., 2006). By contrast, alterations in CD54 (ICAM-1) and CXCR1 also allowed us to define other phenotypes including freshly isolated (CD54^{low}, CXCR1^{high}) and N-formylmethionyl-leucyl-
phenylalanine (fMLP)-activated (CD54\textsuperscript{low}, CXCR\textsuperscript{1low}) neutrophils. The fact that the apoptosis of rTEM neutrophils is delayed is one of the supporting pieces of evidence that these neutrophils are functionally distinct from naïve circulating neutrophils and tissue infiltrating neutrophils. The question of whether rTEM neutrophils preserve their potentials of generating chlorinated lipids upon MPO/HOCl/RCS system awaits future studies to answer. More insights on how and where rTEM neutrophils exert their actions may also be obtained by comparing the effects between tissue samples collected from SCLP and CLP groups.
i. References


type 1 toward urokinase type plasminogen activator. *Biochim Biophys Acta Gen Subj.* 1201, 217-222.


i. Appendix I-Materials and methods

I. In vitro studies in HIMVEC to examine inflammatory responses to chlorinated lipids

Mesenteric endothelial cell culture

Human intestinal mesenteric vascular endothelial cells (HIMVEC) were grown in EGM-2MV medium (Lonza, Walkersville, MD) and maintained at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. Cells were incubated with chlorinated lipids (2-ClHDA or 2-ClPA, 10 µmol/L) or non-chlorinated lipids (HDA or PA, 10 µmol/L). 2-ClPA was synthesized and prepared as described previously using hexadecanoic acid as precursor (Wacker et al., 2013). 2-ClHDA was prepared by treating 1-O-hexadec-1’-enyl-glycerol-3-phosphocholine (100 mg) with freshly prepared hypochlorous acid (final concentration 1.5 mM) in phosphate buffer (pH 4) for 5 min at 37°C (Wacker et al., 2013). All other lipids were obtained from NuChek Prep (Elysian MN). All in vitro studies described below were repeated with at least four separate cell cultures.

Leukocyte adherence to cultured endothelium

Adherence of platelets to HIMVEC was assessed as previously described. Briefly, HIMVEC were grown to confluence in a 12-well plate. Cells were treated with 10 µM BSA-conjugated lipids in growth media for 30 min. Leukocytes were isolated from healthy volunteers. Five hundred microliters (4 x 10⁶ cells/mL) were subsequently added to HIMVEC and incubated for 20 min. Unbound leukocytes were washed away with three PBS rinses. The remaining leukocytes were lysed with 0.2% Triton X-100 and scraped into an Eppendorf tube. Tubes were briefly sonicated to ensure complete lysis of cells. To measure myeloperoxidase, 400 µL cell lysate was transferred
to a test tube containing phosphate buffer, Hank’s buffer with BSA, 1, 9-dimethyl-
methylene blue, and 0.5% hydrogen peroxide. Samples were incubated for 15 min at
room temperature. Sodium azide (1%) was added to stop the reaction. Absorbance was
measured at 460 nm.

Platelet adherence to cultured endothelium

Adherence of platelets to HIMVEC was assessed as previously described. Briefly,
HIMVEC were grown to confluence in a 24-well plate. Cells were treated with 10 µM
bovine serum albumin (BSA)-conjugated lipids in growth media for 30 min. Platelets
were isolated from healthy volunteers. Platelets were stained with Calcein-AM (2.5
µmol/L) for 15 min at 37°C in the dark. Fluorescence-labelled platelets were
subsequently added to HIMEC and incubated for 20 min at 37°C. Unbound platelets were
washed away with three PBS rinses. The remaining platelets were lysed in lysis buffer for
10 min, and fluorescence was measured with a plate reader (excitation at 492 nm,
emission at 535 nm).

Resistance measurements in HIMVEC

Resistance measurements in mesenteric endothelial cells was performed as previously
described (Marentette et al., 2015). HIMVEC were grown to confluence on Transwell
inserts, and then incubated with 2-ClHDA or 2-CIPA (10 µmol/L) or in media alone.
Changes in electrical resistance were measured over time using an epithelial volt
ohmmeter.

II. In vivo studies in HIMVEC to examine inflammatory responses to chlorinated lipids

Animals

See materials and methods in Chapter II.
Chlorinated lipids superfusion

Chlorinated lipids were kindly provided by Dr. David A. Ford from Center for Cardiovascular Research, Saint Louis University. Anesthetized rats as previously described, then exteriorized the mesentery and superfused with physiological salt solutions containing chlorinated lipids (2-ClHDA or 2-ClPA, 10 µmol/L,) versus non-chlorinated lipid (HDA or PA, 10 µmol/L). Inflammatory responses were evaluated at baseline, 30 min, 60 min, and 90 min upon superfusion.

Assessing inflammatory responses in mesentery to lipids

In our *in vivo* experiments, leukocyte- and platelet -endothelial cell interactions, mast cell activation, albumin extravasation, and reactive oxygen species (ROS) production were quantified in rat mesenteries superfused with HDA, 2-ClHDA, PA and 2-ClPA, according to the procedures described below.

Leukocyte- and platelet-endothelial adhesive interactions

See materials and methods in *Chapter II*.

Mast cell activation

See materials and methods in *Chapter II*.

Albumin leakage

See materials and methods in *Chapter II*.

ROS production

In the second batch of rats, the oxidant-sensitive fluorescent probe dihydrorhodamine (DHR 123) was added to BBS (10 µM) to estimate ROS generation in mesenteric postcapillary venular walls. In brief, DHR 123 in BBS was superfused over mesentery...
and the mesentery was scanned for the baseline assessment, then the perfusate was
switched to one containing DHR 123 and either chlorinated lipid or non-chlorinated lipid
solutions. At each time point, 10 single-unbranched postcapillary venules (20-50 μm in
diameter and 100 μm in length) were observed and images were acquired. The
fluorescence intensity was measured on the venular wall in 5 regions of interest (25 μm in
diameter) (Steiner et al., 2003). Since the baseline fluorescence intensity varies
depending on the animal, the ratio of the fluorescence intensity at each time point to the
baseline was calculated as DHR fluorescence ratio (Zuidema and Korthuis, 2015).

*Immunohistochemical staining for MPO*

See materials and methods in *Chapter II.*

*Tissue MPO activity*

Mucosal MPO activity was measured in biopsies obtained from rat small intestine
(jejunum) collected at the end of superfusion period, with use of a fluorescence assay kit
(Cell Technology, Mountain View, CA). Samples were prepared as per kit
manufacturer’s instructions and MPO activity in the samples was quantified by adding
the kit detection reagent and measuring the resulting fluorescence (excitation at 530 nm;
emission at 590 nm) (Liu et al., 2012). Values were normalized to sample protein content
and MPO activity was expressed as milliunits per milligram protein.

*Statistical analysis*

MetaMorph (Nashville, TN) was used to estimate the inflammatory responses from
intravital microscopy. Graphpad Prism 6.0 was used to generate all statistical values.
Data were analyzed using one-way ANOVA and two-way ANOVA followed by post hoc
analysis using Newman-Keuls test or Fisher’s LSD test. Data are presented as means ±
SEM. Differences were regarded as significant at $P < 0.05$ and highly significant at $P < 0.01$. 
ii. Appendix I-Tables and figures

Figure Appx I-1. The changes in adherence to HIMVEC exposure to lipids in vitro

PMN and platelet adherence to human intestinal mesenteric vascular endothelial cells (HIMVEC) occurred when incubated with lipids. (A) HDA promotes neutrophil firm adhesion, whereas 2-ClHDA and 2-CIPA scale up this effect to a greater extent. As follows: 5.55 ± 0.34 in HDA group; 13.55 ± 1.38 in HDA; 25.4 ± 1.63 in 2-ClHDA; 7.13
± 0.69 in PA; and 32.4 ± 2.65 in 2-CIPPA. (B) PA induced platelet adhesion; 2-ClHDA and 2-CIPA indeed further augmented the firm adhesion to HIMVEC in vitro: 170.17 ± 14.33 (control), 204.67 ± 5.78 (HDA), 313.67 ± 16.22 (2-ClHDA), 300.67 ± 7.46 (PA), and 430.33 ± 17.94 (2-CIPA). n=8. **, p < 0.01.
Table Appx I-1. Leukocyte rolling patterns to lipids superfusion

<table>
<thead>
<tr>
<th>Trait</th>
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<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA</td>
<td>130.36 ± 31.33</td>
<td>180.33 ± 46.03</td>
<td>166.30 ± 31.18</td>
<td>214.13 ± 37.32</td>
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<tr>
<td>2-ClHDA</td>
<td>102.22 ± 22.70</td>
<td>334.77 ± 34.35**</td>
<td>351.95 ± 39.10**</td>
<td>388.23 ± 30.03**</td>
</tr>
<tr>
<td>PA</td>
<td>98.11 ± 22.49</td>
<td>130.59 ± 14.17</td>
<td>127.53 ± 32.99</td>
<td>135.16 ± 22.86</td>
</tr>
<tr>
<td>2-ClPA</td>
<td>113.52 ± 18.25</td>
<td>429.12 ± 70.31*</td>
<td>611.11 ± 59.58**</td>
<td>826.73 ± 260.40**</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean ± SEM. n=6. *, p < 0.05, **, p < 0.01.

Figure Appx I-2. Chlorinated lipids promote leukocyte rolling in vivo.

The estimation of leukocytes rolling (per mm² per minute) in postcapillary venules was increased by 2-ClHDA or 2-ClPA superfusion in rat mesentery, as opposed to HDA or PA challenge.
Table Appx I-2. Leukocyte adhesion patterns to lipids superfusion

<table>
<thead>
<tr>
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<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA</td>
<td>1.95 ± 1.95</td>
<td>4.66 ± 2.95</td>
<td>4.34 ± 2.87</td>
<td>10.57 ± 3.24</td>
</tr>
<tr>
<td>2-ClHDA</td>
<td>3.20 ± 3.20</td>
<td>15.15 ± 9.87</td>
<td>9.50 ± 5.63</td>
<td>10.00 ± 0.23</td>
</tr>
<tr>
<td>PA</td>
<td>1.53 ± 1.53</td>
<td>1.63 ± 1.63</td>
<td>1.29 ± 1.29</td>
<td>6.35 ± 2.85</td>
</tr>
<tr>
<td>2-ClPA</td>
<td>3.08 ± 2.23</td>
<td>17.99 ± 6.54</td>
<td>9.93 ± 6.3</td>
<td>30.22 ± 11.84*</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean ± SEM. n=6. *, p < 0.05.

Figure Appx I-3. Chlorinated lipids tend to increment leukocyte adhesion in vivo.

The evaluation of leukocyte adhesion (per mm² per 30 seconds) reveals leukocyte firm adhesion in response to chlorinated lipids (2-ClHDA and 2-ClPA), and non-chlorinated lipids (HDA and PA) superfusion in postcapillary venules. Indeed, superfusion of rat mesenteries with physiological salt solutions containing either 2-ClHDA or 2-ClPA promoted marked increases in the numbers of firmly adherent leukocytes, especially 90 min after 2-ClPA.
Table Appx I-3. Platelet rolling patterns to lipids superfusion

<table>
<thead>
<tr>
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<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA</td>
<td>147.80 ± 82.88</td>
<td>102.31 ± 71.98</td>
<td>84.74 ± 31.32</td>
<td>85.94 ± 42.61</td>
</tr>
<tr>
<td>2-ClHDA</td>
<td>106.18 ± 3.20</td>
<td>346.21 ± 58.90*</td>
<td>233.28 ± 5.63</td>
<td>229.60 ± 105.99</td>
</tr>
<tr>
<td>PA</td>
<td>143.96 ± 26.38</td>
<td>92.77 ± 71.98</td>
<td>44.02 ± 35.15</td>
<td>92.34 ± 92.40</td>
</tr>
<tr>
<td>2-ClPA</td>
<td>89.54 ± 33.74</td>
<td>370.28 ± 123.76**</td>
<td>333.08 ± 63.67*</td>
<td>358.87 ± 222.39**</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean ± SEM. n=8 except n=6 in HDA and n=5 in PA. *, p < 0.05, **, p < 0.01.

Figure Appx I-4. Chlorinated lipids facilitate platelet rolling in vivo.

The platelet rolling was evaluated the same way as leukocyte rolling that, the multitude of rolling platelet was determined in answer to chlorinated lipids (2-ClHDA and 2-ClPA), and non-chlorinated lipids (HDA and PA) superfusion in postcapillary venules. Upward trends were observed in both chlorinated lipids groups, as opposed to their corresponding controls (data in Table Appx I-3).
Table Appx I-4. Platelet adhesion patterns to lipids superfusion

<table>
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<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA</td>
<td>11.11 ± 7.88</td>
<td>14.95 ± 5.57</td>
<td>16.00 ± 7.16</td>
<td>28.98 ± 14.28</td>
</tr>
<tr>
<td>2-ClHDA</td>
<td>11.48 ± 5.99</td>
<td>38.98 ± 10.31*</td>
<td>31.54 ± 5.94</td>
<td>42.77 ± 9.58**</td>
</tr>
<tr>
<td>PA</td>
<td>15.59 ± 13.92</td>
<td>10.17 ± 13.02</td>
<td>17.03 ± 17.60</td>
<td>26.46 ± 16.27</td>
</tr>
<tr>
<td>2-ClPA</td>
<td>5.25 ± 5.83</td>
<td>36.79 ± 25.97*</td>
<td>67.35 ± 22.48**</td>
<td>58.50 ± 10.89**</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean ± SEM. n=6 except n=8 in 2-ClHDA and n=7 in 2-ClPA. *, p < 0.05; **, p < 0.01.

Figure Appx I-5. Chlorinated lipids boost platelet adhesion in vivo.

The platelet firm adhesion in postcapillary venules was estimated in chlorinated lipids (2-ClHDA and 2-ClPA), and non-chlorinated lipids (HDA and PA) groups, respectively. As expected, chlorinated lipids tend to enhance platelet adhesion compared to the non-chlorinated lipids, and the significant difference were marked in Table Appx I-4.
Table Appx I-5. Mast cell activation patterns to lipids superfusion

<table>
<thead>
<tr>
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<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA</td>
<td>0.82 ± 0.31</td>
<td>2.82 ± 0.30</td>
<td>3.42 ± 0.27</td>
<td>4.05 ± 0.28</td>
</tr>
<tr>
<td>2-ClHDA</td>
<td>1.00 ± 0.30</td>
<td>8.03 ± 0.95**</td>
<td>8.22 ± 0.89**</td>
<td>7.71 ± 1.10**</td>
</tr>
<tr>
<td>PA</td>
<td>1.08 ± 0.39</td>
<td>3.53 ± 0.65</td>
<td>4.15 ± 0.52</td>
<td>4.42 ± 0.44</td>
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<tr>
<td>2-ClPA</td>
<td>0.45 ± 0.45</td>
<td>7.21 ± 1.62**</td>
<td>8.53 ± 2.11**</td>
<td>9.17 ± 2.18**</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean ± SEM. n=6. **, p < 0.01.

Figure Appx I-6. Mast cell activation changes with chlorinated lipids superfusion.

The number of activated mast cells per field in response to HDA, 2-ClHDA, PA and 2-ClPA superfusion in mesenteric membrane was quantified. Both 2-ClHDA and 2-ClPA elicited mast cell activation within 30 min of exposure and remained elevated for the remainder of the experimental protocol. Mesenteric superfusion with non-chlorinated HDA and PA also showed similar effects but to levels lower than that evoked by the chlorinated lipids. At each time point for every animal, 10 fields (20x) were inspected.
Table Appx I-6. Reactive oxygen species (ROS) patterns to lipids challenge

<table>
<thead>
<tr>
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<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA</td>
<td>1.00 ± 0.00</td>
<td>1.07 ± 0.13</td>
<td>1.23 ± 0.10</td>
<td>1.33 ± 0.16</td>
</tr>
<tr>
<td>2-ClHDA</td>
<td>1.00 ± 0.00</td>
<td>1.72 ± 0.14*</td>
<td>2.08 ± 0.13**</td>
<td>2.59 ± 0.26**</td>
</tr>
<tr>
<td>PA</td>
<td>1.00 ± 0.00</td>
<td>1.20 ± 0.27</td>
<td>1.51 ± 0.48</td>
<td>1.34 ± 0.28</td>
</tr>
<tr>
<td>2-ClPA</td>
<td>1.00 ± 0.00</td>
<td>2.48 ± 0.91*</td>
<td>2.28 ± 0.42*</td>
<td>2.41 ± 0.33*</td>
</tr>
</tbody>
</table>

Note: Data are normalized to baseline and presented as mean ± SEM. n=6. *, p < 0.05, **, p < 0.01.

Figure Appx I-7. Chlorinated lipids further ROS production in mesentery.

The DHR 123 fluorescence intensity along the postcapillary venule wall was evaluated, and normalized to baseline (0 min), yielding a ratio indicating ROS production responding to HDA, 2-ClHDA, PA, and 2-ClPA, respectively. 10 mesenteric venules were randomly chosen, and for each of them, 5 regions of interest (25 µm in diameter) along the venule wall were quantified. Superfusion with chlorinated lipids increased DHR fluorescence in mesenteric postcapillary venules; on the contrary, DHR fluorescence for mesenteric exposure to HDA and PA were not affected throughout the observation period.
Table Appx I-7. Resistance change patterns to lipids challenge

<table>
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<tr>
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<th>3 h</th>
<th>6 h</th>
<th>9 h</th>
<th>12 h</th>
<th>15 h</th>
<th>18 h</th>
<th>21 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>-5.3 ± 3.8</td>
<td>0 ± 1.5</td>
<td>4.7 ± 1.7</td>
<td>-0.7 ± 5.2</td>
<td>5.3 ± 2.7</td>
<td>1 ± 6.7</td>
<td>3.3 ± 3.2</td>
<td>3 ± 6</td>
</tr>
<tr>
<td>HDA</td>
<td>0.0 ± 0.0</td>
<td>-11.3 ± 4.7</td>
<td>-14.4 ± 4.6</td>
<td>-12.7 ± 8.9</td>
<td>-22.6 ± 6.4</td>
<td>-21.7 ± 4.1**</td>
<td>-22.3 ± 7.5*</td>
<td>-27 ± 3.2**</td>
<td>-30.3 ± 6.9**</td>
</tr>
<tr>
<td>2-CIHDA</td>
<td>0.0 ± 0.0</td>
<td>-32.3 ± 6.9</td>
<td>-34 ± 2.3</td>
<td>-36 ± 2.1*</td>
<td>-47 ± 1.8*</td>
<td>-57 ± 2.0*</td>
<td>-62 ± 3.5**</td>
<td>-63.7 ± 10.9**</td>
<td>-62.7 ± 10.9**</td>
</tr>
<tr>
<td>PA</td>
<td>0.0 ± 0.0</td>
<td>-12 ± 2.1</td>
<td>-13 ± 3.6</td>
<td>-2.3 ± 6.6</td>
<td>-11 ± 4.4</td>
<td>-14.7 ± 6.6*</td>
<td>-12.3 ± 10.1</td>
<td>-12.3 ± 5.8*</td>
<td>-12.7 ± 6.4</td>
</tr>
<tr>
<td>2-ClPA</td>
<td>0.0 ± 0.0</td>
<td>-15.3 ± 4.3</td>
<td>-16.3 ± 7.7</td>
<td>-24 ± 10.6</td>
<td>-34.7 ± 1.3</td>
<td>-42.7 ± 5.4**</td>
<td>-53 ± 4.2**</td>
<td>-59 ± 6.8**</td>
<td>-53 ± 4**</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean ± SEM. n=6. At each indicated time points: *, p < 0.05, **, p < 0.01 when compared to control; ^, p < 0.05 and ^^, p <0.01 between chlorinated lipids and non-chlorinated lipids groups.

Figure Appx I-8. The changes in electrical resistance of HIMVEC exposure to lipids

Changes in endothelial permeability (as reflected by reductions in electrical resistance across HIMVEC monolayers) induced by chlorinated lipids exposure. We detected a significant decrease in electrical resistance in endothelial cells following exposure to the various lipids (HDA, 2-CIHDA, PA, and 2-ClPA) compared to control, with much larger reductions in monolayer electrical resistance exhibited by HIMVC exposed to 2-CIHDA and 2-ClPA compared to the measurements obtained in the HDA and PA exposure groups, respectively.
Table Appx I-8. Albumin leakage (permeability) patterns to lipids challenge

<table>
<thead>
<tr>
<th>Trait</th>
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<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA</td>
<td>1.00 ± 0.00</td>
<td>0.98 ± 0.05</td>
<td>0.97 ± 0.15</td>
<td>1.07 ± 0.17</td>
</tr>
<tr>
<td>2-ClHDA</td>
<td>1.00 ± 0.00</td>
<td>2.13 ± 0.49*</td>
<td>2.69 ± 0.53*</td>
<td>3.21 ± 0.60**</td>
</tr>
<tr>
<td>PA</td>
<td>1.00 ± 0.00</td>
<td>1.11 ± 0.21</td>
<td>1.08 ± 0.16</td>
<td>1.03 ± 0.04</td>
</tr>
<tr>
<td>2-CIPPA</td>
<td>1.00 ± 0.00</td>
<td>3.19 ± 0.00**</td>
<td>3.56 ± 0.19**</td>
<td>3.99 ± 0.17**</td>
</tr>
</tbody>
</table>

Note: Data are normalized to baseline and presented as mean ± SEM. n=6. *, p < 0.05, **, p < 0.01.

Figure Appx I-9. FITC-albumin leakage ensuing chlorinated lipids application.

The fluorescence intensity within mesenteric postcapillary venules (Iv) and in perivenular interstitium (Ip) were quantified upon the application of HDA, 2-ClHDA, PA and 2-CIPPA. 10 postcapillary venules in mesentery were randomly chosen and 5 regions of interest (r=12.5 mm) inside and outside of the venule wall were computed. The ratio of Ip/Iv was calculated at each pair of corresponding spots, and the mean ratio at each time point was normalized to baseline. Albumin leakage was increased with chlorinated lipid challenge; however, it was not detected before lipids treatment in all groups, and this situation persisted throughout the observation period in mesenteries superfused with HDA and PA for 90 min.
Figure Appx I-10. MPO staining and activity in small intestine regarding lipids challenge.

(A) Representative pictures from IHC staining for MPO. Arrowheads indicate positive staining (brown) cells. Scale bars=50 μm. (B) Quantification of positive staining cells for MPO. 2-ChHDA and 2-ChPA indeed increased MPO staining, as follows: 12.98 ± 1.25 for HDA; 40.28 ± 4.75 for 2-ChHDA; 12.53 ± 1.6 for PA; and 37.07 ± 5.46 for 2-ChPA. For each animal, 10 fields under microscope (40x) focused on submucosa were randomly
selected. (C) Tissue MPO activity was analyzed by measuring resulting fluorescence at 530 nm (excitation) and 590 nm (emission). The measurements were repeated for three times and mean values were normalized to sample protein content. Significant increase of MPO activity was captured in 2-ClHDA and 2-ClPA, and the data for four groups are listed: 720.24 ± 102.31 (HDA); 1568.40 ± 409.18 (2-ClHDA); 818.86 ± 95.97 (PA); 1434.46 ± 243.82 (2-ClPA). Data are presented as mean ± SEM. n=6. *, p < 0.05, **, p < 0.01.
iii. Appendix I-References


iv. Appendix II-Materials and methods

Adhesion molecule cell surface expression

HIMVEC grown to confluence in 16-mm culture dishes, were incubated with 10 µmol/L chlorinated lipids (2-ClHDA or 2-ClPA) versus non-chlorinated lipid (HDA or PA), or in media alone for indicated times at 37°C in 95% O₂-5% CO₂, respectively. At the end of incubation, buffer was quickly removed, and cells were immediately fixed with ice-cold 1% paraformaldehyde and incubated overnight at 4°C. Cells were washed three times with phosphate-buffered saline (PBS) and then blocked with Tris-buffered saline-Tween supplemented with 0.8% BSA (wt/vol) and 0.5% fish gelatin (wt/vol) for 1 hr at 24°C. Appropriate primary antibody (1: 50) was used before treatment with horseradish peroxidase-conjugated secondary antibody (1: 5000). Subsequently, each well was incubated in the dark with the 3, 3’, 5, 5’-tetramethylbenzidine liquid substrate system. Reactions were stopped by the addition of sulfuric acid, and color development was measured with a microtiter plate spectrophotometer at 450 nm.
v. Appendix II-Tables and figures

Table Appx II-1. Adhesion molecule patterns to lipids superfusion

<table>
<thead>
<tr>
<th>Trait</th>
<th>Control</th>
<th>HDA</th>
<th>2-ClHDA</th>
<th>PA</th>
<th>2-CIPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin</td>
<td>0.11 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.41 ± 0.04</td>
<td>0.39 ± 0.05</td>
<td>1.08 ± 0.19</td>
</tr>
<tr>
<td>E-selectin</td>
<td>0.12 ± 0.01</td>
<td>0.48 ± 0.06</td>
<td>0.69 ± 0.13</td>
<td>0.19 ± 0.02</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0.13 ± 0.02</td>
<td>0.33 ± 0.04</td>
<td>0.65 ± 0.08</td>
<td>0.23 ± 0.03</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>0.12 ± 0.02</td>
<td>0.35 ± 0.04</td>
<td>0.49 ± 0.01</td>
<td>0.28 ± 0.05</td>
<td>0.43 ± 0.02</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean ± SEM.

Figure Appx II-1. Expression of adhesion molecules on cell surface to lipids

HIMVEC exposed to chlorinated and non-chlorinated lipids, and expression of adhesion molecules on cell surface was measured at indicated times of incubation. P-selectin, E-selectin, ICAM-1 and VCAM-1, were increased by exposure to the chlorinated lipids (2-ClHDA and 2-CIPA), while the non-chlorinated lipids (HDA and PA) exerted more modest effects relative to control. Significant increases in P-selectin and E-selectin expression were detected when measured after 30 minutes and 1 hour of chlorinated lipids exposure, and in ICAM-1 and VCAM-1 expression after 4 hours of exposure. n=6. *p < 0.05 and **
$p < 0.01$ compared to control; $^5 p < 0.05$ and $^{55} p < 0.01$ between 2-CIPA and PA, 2-ClHDA and HDA groups at indicated time point.
VITA

Hong Yu was born in December 1987 in Sichuan, China. After graduating from medical school, she attended the graduate school at West China Medical Center of Sichuan University as a Master student in September 2010. In 2013, she finished her three-year clinical and research training in the Department of Anesthesiology and completed her master’s degree in China.

In the summer of 2015, she attended University of Missouri to pursue a master’s degree in the Department of Medical Pharmacology and Physiology. After joining in Dr. Ronald J. Korthuis’s lab, where she completed her first project in chlorinated lipids in the spring of 2017, she extended her studies to the regulation of sepsis with Dr. Ronald J. Korthuis as a Ph.D. student and expected to graduate in the spring of 2020. Upon her graduation, she will continue her research training in United States.