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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Protease Activity in Mesenteric Lymph Following Splanchnic Arterial Occlusion

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Bioengineering

by

Michael D. Richter

Committee in Charge:

Professor Geert W. Schmid-Schönbein, Chair
Professor Antonio De Maio
Professor Michael Heller

2012

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Chair

University of California, San Diego
2012

DEDICATION

I dedicate this thesis to my family.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
ELAM	Endothelial leukocyte adhesion molecule
ICAM	Inter-cellular adhesion molecule
HS	Hemorrhagic shock
MMP	Matrix metalloproteinase
MOF	Multiple organ failure
PAR-2	Protease-activated receptor 2
SAO	Splanchnic arterial occlusion
VEGFR-3	Vascular Endothelial Growth Factor Receptor 3

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ABSTRACT OF THE THESIS

Protease Activity in Mesenteric Lymph Following Splanchnic Arterial Occlusion

by

Michael D. Richter

Master of Science in Bioengineering

University of California, San Diego, 2012

Professor Geert W. Schmid-Schönbein, Chair

The lymphatics play a key role in the pathogenesis of multiple organ failure following circulatory shock. Understanding the cause of this organ dysfunction is a fundamentally unsolved problem in medicine. Current results show that biologically active molecules produced in the gut enter the circulation via the mesenteric lymph duct, and induce neutrophil activation, increased vascular permeability, and acute lung injury. Despite several attempts to determine the protein composition of mesenteric lymph, the

factors responsible for this biological activity and specifically the organ damage are unknown.

The aim of this study was to investigate the level and activity of pancreatic digestive proteases in post-shock lymph, which could be derived from the intestine and which are a potentially damaging mediator. Male Wistar rats were subjected to laparotomy, followed by mesenteric lymph duct cannulation and one hour of lymph fluid collection before shock. Five animals then underwent one hour of splanchnic arterial occlusion followed by reperfusion, while sham shock animals remained perfused. Post-shock lymph was collected for three hours, with the samples aliquoted every hour. Protease activity in the lymph was analyzed using a fluorescently quenched casein substrate as an indicator for general protease activity and separately charge-changing fluorescently quenched peptide substrates specific to pancreatic digestive proteases (trypsin, chymotrypsin).

These measurements showed trypsin and chymotrypsin activity in the lymph, and the presence of trypsin was confirmed using Western analysis. Over the time course we see a base level of protease activity in the mesenteric lymph that increases following gut ischemia and reperfusion. These results indicate that during shock digestive protease escape from the splanchnic bed, raising the possibility that they are involved in the systemic injury following shock. Supported by NIH grant GM-85072.

I. INTRODUCTION

A. INTRODUCTION TO SHOCK AND THE AUTODIGESTION HYPOTHESIS

Circulatory shock is a life-threatening medical condition caused by a lack of perfusion with inadequate oxygen delivery to the body's vital organs and a progression of cell dysfunctions. There are a number of causes for the condition, including trauma, burns, hypovolemia, blood flow obstruction, myocardial dysfunction, and sepsis. Of these, hypovolemia resulting from trauma injury is the most clinically relevant, as it is the leading cause of death of humans under the age of 44 years (1).

A common cause of death for patients who survive the first 24 hours following their initial injury is multiple organ failure (MOF), with mortality rates in excess of 40% (2). MOF is characterized by a dysfunction of organs that were not involved with the original injury, and is most frequently associated with lung, heart, liver, intestine, and kidney damage. Organ failure is also accompanied by an unregulated inflammatory response, which has been linked to a number of factors that can cause blood cytotoxicity (3). However, the pathogenesis of MOF and systemic inflammation following shock remains a fundamentally unsolved problem in medicine, and advances in treatment will depend on a greater understanding of the mechanisms underlying the problem.

Originally, physicians considered translocation of bacteria from the intestine the most likely source of organ dysfunction. While sepsis often occurs in patients with MOF, there is little evidence showing a causative link, and the theory has largely been unconfirmed in most patients (4,5). Over the past decade, an alternative theory termed the *Autodigestion Hypothesis* has taken precedence as a potential explanation for shock-

induced organ damage. The theory states that during ischemia after blood loss, the mucosal barrier that separates the intestinal lumen from the intestinal wall breaks down (6). This allows proteases that are synthesized by the pancreas and released into the lumen for food digestion to break down the body's own tissues, causing necrosis and the potential release of inflammatory mediators into the circulation. (5) This is supported by studies that show that intestinal tissues in the presence of proteases are capable of causing endothelial cell and blood cell activation and cytotoxicity (7). Further, this cytotoxicity can be prevented by the use of broad-spectrum pancreatic digestive enzyme inhibitors, which corresponds to observations in rat models that protease inhibitors injected into the intestinal lumen before an episode of gut ischemia reperfusion (IR) reduces subsequent organ damage (8). Other successful treatments that support the theory include flushing the intestine before shock and ligating the pancreatic duct to reduce the luminal protease concentration (9).

The *Autodigestion Hypothesis* has been the subject of recent clinical studies. One such study reported the successful use of an inhibitor treatment on a heart transplant patient suffering from septic shock. Administration of a synthetic digestive protease inhibitor by enteral feeding rapidly reversed markers of systemic inflammation (10). However, the precise role of proteases in the pathogenesis of MOF is still unclear, and it is unknown whether they enter into the circulation and have an impact outside of the intestinal wall.

B. ROLE OF THE LYMPHATIC SYSTEM IN MULTIPLE ORGAN FAILURE

Efforts to understand how gut injury and intestinal permeability contribute to distant organ dysfunction have repeatedly pointed to the lymphatic system. Inflammatory cytokines originating in the gut use the mesenteric lymphatics as a transport system to reach the circulatory system (11). Many studies show the ability of mesenteric lymph fluid collected after a period of intestinal IR to recreate many of the symptoms of shock and MOF. These include neutrophil activation, abnormal cardiac myocyte function, increased endothelial cell permeability, reduced red blood cell deformability, and bone-marrow dysfunction (12, 13, 14). *In-vivo* experiments also show that cross-transfusion of postshock mesenteric lymph into a naïve rat induces acute lung injury, and ligation of the main lymph duct exiting the mesentery abrogates organ damage and systemic inflammation (15, 16).

There are several physiological explanations for the importance of the lymphatic system in shock. Lymph flowing from the intestine travels through the mesenteric lymph duct and into the subclavian vein via the thoracic duct. This venous blood flows directly into the heart and lungs, providing harmful mediators produced in the intestine a direct pathway that does not travel through the liver, where such products may be absorbed and/or broken down (2, 17). Also, the lymphatic vessels in the intestine that normally transport fats and nutrients from the intestinal lumen into the circulation are ideally placed to transport byproducts of autodigestion. The villi in the intestine have prelymphatic channels that connect with a network of lymph vessels in the mucosal layer. A separate system of lymphatics exists for draining the mucosal muscle layer, and the two networks join at contractile vessels on the mesenteric border (18). The structural

breakdown and necrosis within intestinal wall after IR would allow these channels to act as a conduit for accumulated extracellular fluid to exit the intestine and enter the circulation (5).

While it is theoretically possible for cytotoxic mediators to enter the bloodstream via the microcirculation rather than the lymphatics, measurements suggest this is not likely the case. One study that collected blood from the portal circulation along with lymph fluid concluded that while the lymph induced endothelial cell permeability and lung injury, the portal blood did not (19). This reinforces the lymphatic system as a key transport pathway between intestinal IR and organ damage.

C. PROTEOME OF POSTSHOCK MESENTERIC LYMPH

Several research groups have tried to identify the factors in lymph responsible for organ injury. Recent efforts have centered on using mass spectroscopy to obtain a proteome of preshock and postshock mesenteric lymph, and analyzing the proteins with a significant change in concentration between the two groups (20, 21, 22). While none of these studies give conclusive statements regarding the specific inflammatory mediators, they have highlighted several possibilities. Many of the upregulated proteins exhibit immunomodulatory effects or play a role in innate immunity, supporting the hypothesis that systemic inflammation contributes to MOF (22). They also show an increase in amylase and carboxypeptidase A2 concentration, supporting the hypothesis of Ishimaru *et al* that pancreatic proteases could pass through the intestinal mucosal barrier and into the submucosal space (20, 23).

However, it is important to note the inability of these proteomic measurements to fully profile the role of proteases in postshock inflammation and organ damage. A typical mass spectroscopy protocol requires the addition of exogenous trypsin to fragment the protein, impairing the detection of this major pancreatic enzyme (5). It is also important to distinguish enzyme concentration from activity. The mechanistic significance of protease activity in lymph would almost certainly correspond to changes in activity (23). Simply looking at concentration changes can be misleading. For example, proteomic studies also report the downregulation of several protease inhibitors, including alpha-2-Macroglobulin and alpha-1-Inhibitor 3, both of which are capable of inhibiting trypsin, chymotrypsin, and other pancreatic proteases (21, 24, 25). It is possible that a change in inhibition induces increased protease activity rather than a change in concentration (21).

Efforts to translate this analytic technique to the clinic have supported the findings of rat studies. Lymph collected after various episodes of shock or myocardial infarction activated neutrophils, and displayed a number of proinflammatory activities. Patients displayed an increased abundance of hemolysis markers, extra-cellular matrix components, and proteins associated with organ damage. The study also confirmed the presence of secreted proteases and protease inhibitors, though not the specific enzymes exhibited in rodent studies (26).

D. SERINE PROTEASES AND RECEPTOR CLEAVAGE

There is evidence that an increase in protease activity may play a role in organ failure following intestinal IR. There are already several studies that establish protease activity in plasma following shock, one of which found that pretreating animals with a

protease inhibitor in the intestinal lumen reduced plasma activity during reperfusion (27). The plasma, peritoneal fluid, and lung homogenate of postshock animals also displays elevated levels of caseinolytic, trypsin-, chymotrypsin-, elastase-, and MMP-like activity, with immunoblots confirming the presence of pancreatic trypsin in each tissue sample. Upregulation of MMP-9 was also measured in plasma, peritoneal fluid, heart, liver, and lung tissue, which is noteworthy considering MMPs can be activated by serine proteases (28). Clinically, plasma samples from shock patients have shown elevated levels of amylase and lipase activity (29). These results support the idea that proteases originating in the intestinal lumen may act as shock mediators and may be targets for potential clinical therapies.

Receptor cleavage in vital organs is further evidence for a role of proteases. Increased vascular permeability is a well-documented symptom of circulatory shock, and can often be attributed to the failure of inter-cellular adhesion molecules (ICAM). Both free ICAM-1 and endothelial leukocyte adhesion molecule 1 (ELAM-1) have been detected following physiological shock, as have circulating endothelial cells (30,31). Further, unpublished work from the Schmid-Schönbein laboratory shows a correlation between hemorrhagic shock, MMP activity, and receptor cleavage in the lung, and offers a potential mechanism for the development of MOF based on MMP activation.

E. SUMMARY

Shock and the subsequent multiple organ dysfunction is one of the leading causes of death in intensive care units. However, a lack of understanding of the mechanisms behind this condition prevents advances in treatment. Numerous studies over the past two

decades have established the intestine as a primary source of cytotoxic mediators that cause systemic inflammation and MOF, and implicate the lymphatic system as the main transport mechanism carrying these mediators to peripheral tissues. While a number of proteomic studies have sought to identify these mediators, they are largely inconclusive. One theory suggests that proteases released from the intestine may act as mediators for inflammation and organ damage, activating MMPs and cleaving endothelial cell receptors, though no study has thoroughly investigated their presence or activity.

F. OBJECTIVE

Investigate whether proteases act as a potential mediator for inflammation and organ damage following circulatory shock.

G. HYPOTHESIS

1. Lymphatic fluid collected after intestinal ischemia reperfusion has elevated levels of protease activity.
2. Postshock mesenteric lymph contains increased concentrations of pancreatic proteases.

H. SPECIFIC AIMS

1. Develop a method for continuous collection of mesenteric lymphatic fluid from a rat undergoing splanchnic ischemia reperfusion.
2. Investigate the ability of postshock lymph to digest a large, globular protein.

3. Investigate the ability of postshock lymph to digest substrates specific to trypsin- and chymotrypsin-like enzymes.
4. Measure the presence of the following proteins in postshock lymph using Western analysis:
 - a. Trypsin
 - b. Chymotrypsin
 - c. MMP-9
 - d. VEGFR-3

II. Materials and Methods

A. INTRODUCTION

This study uses a rat model of shock to induce physiological conditions that model trauma and hypoperfusion. The protease activity of lymph was determined with *in-vitro* techniques that measure the fluid's ability to digest artificial substrates, simulating its possible behavior *in-vivo*. Therefore, the methods in this study aim to establish a possible mechanistic explanation for shock, rather than generate any clinically relevant findings.

B. SURGICAL PROCEDURE

All animal protocols were reviewed and approved by the University of California, San Diego Animal Subjects committee. Male Wistar rats weighing between 250 and 350 g (Harlan Labs, Indianapolis, IN) were administered general anesthesia with an intramuscular injection of 75 mg/kg ketamine and 4 mg/kg xylazine. Local anesthesia (1% Lidocaine) was delivered followed by cannulation of the left femoral artery and vein with PE 50 tubing. Catheters were filled with heparinized saline (10 mg/ml) to prevent clotting. After a 3 cm midline laparotomy, the duodenum and intestines were removed and placed on a 1 cm platform to the left of the animal and covered with saline-soaked gauze and saran wrap, exposing the base of the mesentery and the superior mesenteric lymph duct. A 1 cm bridge was placed under the animal to further expose the lymph duct, which was then cleared of fat and surface peritoneum using blunt dissection. Silastic

tubing (0.64 mm internal diameter) pre-filled with heparin (10 mg/ml) was drawn through the right abdominal wall using a suture needle and looped under the vena cava using curved tweezers. The superior mesenteric lymph duct was cannulated with the silastic tubing, and held in place with VetBond® tissue glue (3M, St. Paul, MN). The cannulation technique is illustrated in **Figure 1**.

Once lymph fluid reached the end of the cannula, the end was placed inside of a 2 ml tube preloaded with 5 μ l of heparin. Fluid was collected for one hour under the same conditions for every animal. For the splanchnic arterial occlusion (SAO) group (N=5), two micro-clamps were used to occlude the superior mesenteric artery and the celiac artery for the duration of the second hour, while animals in the SHAM group (N=5) remained perfused. After the second hour, the clamps were removed from the SAO animals to begin reperfusion. Lymph fluid was collected continuously for another three hours, and aliquoted every hour. At the end of each hour, samples were centrifuged (1600 g, 4°C, 20 min) to remove cellular debris and excess fat, and the supernatant was stored at -80°C. After the five hours of lymph fluid collection, animals were euthanized (120 mg/kg sodium pentobarbital i.v.).

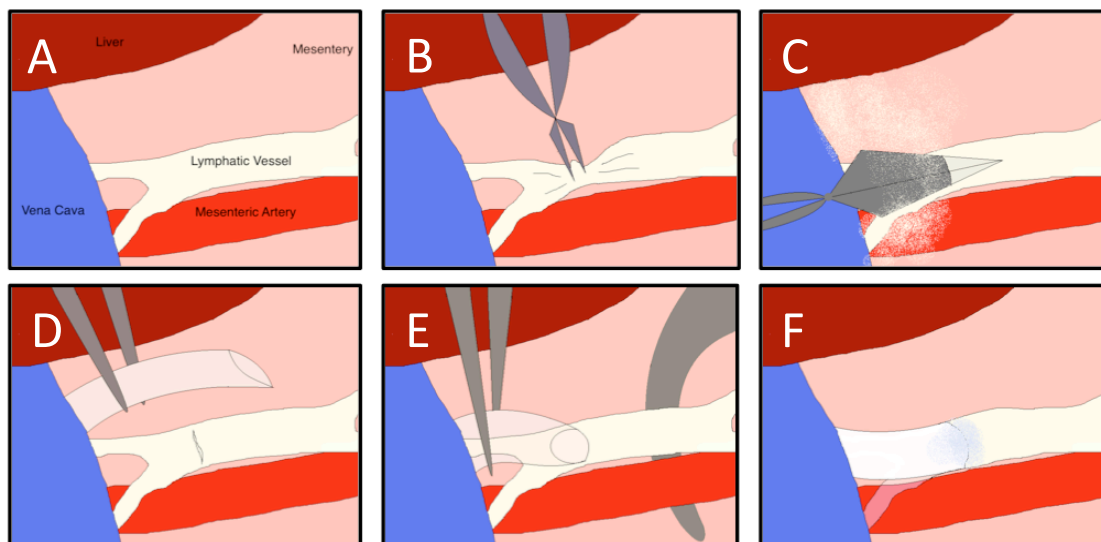


Figure 1: Outline of the technique for cannulation of the superior mesenteric lymph duct with silastic tubing. B. A small lateral incision is made across the top of the vessel using micro-dissection scissors. Fluid released into the abdominal cavity should be cleared with gauze. C. Insertion of a blunt blade of a pair of microscissors into the cut to clear and slightly widen the opening. D. The beveled end of the silastic tubing is placed over the lymph vessel. The tube should sit naturally in place as shown in the picture. E. Curved tweezers lift and hold the lymph vessel in place while the silastic tube is slowly placed into the opening. The tube should not be pushed too far into the vessel, as it diverges soon after entering the mesentery tissue. F. Gauze is used to gently dry the cannulation and a small drop of tissue glue holds the tube in place.

C. PROTEASE ACTIVITY ASSAYS

i. CASEIN ASSAY

To simulate the lymph fluid's ability to digest large globular proteins, we used a casein substrate that is internally quenched with Texas Red fluorophores (589 excitation and 617 emission, Invitrogen, Carlsbad, CA). One part lymph fluid was added to four parts digestion buffer and five parts casein substrate in a black walled clear bottom plate, and each time point was loaded in triplicates. A known quantity of Texas Red (Vector Laboratories, Burlingame, CA) mixed with digestion buffer and lymph was used to

correct for auto-fluorescence of the sample at 617 nm. Three wells were also filled with PBS buffer 9:1 with sample as a control for the fluorescent activity of lymph fluid without substrate. Immediately after loading the samples, the fluorescence was measured every 15 minutes for 2 hours.

ii. CHARGE CHANGING SUBSTRATES

To measure the activity of specific pancreatic enzymes, we used a new electrophoretic method developed by Lefkowitz *et al* (32). The method is superior to colorimetric assays since the lymph often has an opacity (due to light diffraction) that interferes with absorbance measurements. These substrates are small peptide chains that carry opposing charges at each end. When they are cleaved, the end with an attached fluorophore has a net positive charge, making it possible to isolate the cleaved segment with electrophoreses. A trypsin specific substrate (acetyl-N-DGDAGRAGAGK-NH₂) synthesized by Aapptec (Louisville, KY) was labeled on the lysine residue's ϵ amine group with Bodipy FL-SE (Invitrogen, Carlsbad, CA). A similar chymotrypsin specific peptide (acetyl-N-DGDAGYAGLRGAG-diamino ethyl- Bodipy FL) was also synthesized. While the cleavage sites of these substrates are designed for trypsin and chymotrypsin, it is possible for other proteolytic enzymes within those protein families to cleave them at a slower rate. Solution of each substrate (0.5 mg/mL) was combined with 2 μ l of lymph in individual reaction tubes, and allowed to react for one hour. Aliquots of each sample were electrophoresed at 80V for 10 minutes in 4% agarose gel. Gels were quantified using a Story 840 gel scanner, and we integrated over each sample band and

subtracted the background (negative control, substrate+HCl) to obtain the final fluorescence values.

D. WESTERN ANALYSIS

We used immunoblotting to determine the presence and relative concentrations of trypsin, chymotrypsin, MMP-9, and VEGFR-3 in mesenteric lymph. Each lymph sample was denatured by adding it to an equal part of 2 X sample loading buffer (Bio-Rad) containing 0.05% (by volume) 2-mercaptoethanol and boiling for 10 minutes. 5 μ l of the denatured lymph solution was loaded per well into a precast 4-20% gel (Bio-Rad), with a protein ladder and 2 μ l of pancreas homogenate for a trypsin control. Samples were separated and the protein was transferred to a nitrocellulose membrane. Membranes were blocked with 5% bovine serum albumin (BSA), and primary antibodies against pancreatic trypsin (1:1000, sc-137077, Santa Cruz Biotechnology, Santa Cruz, CA), chymotrypsin (1:1000, sc-130007, Santa Cruz Biotechnology, Santa Cruz, CA), MMP-9 (1:1000, ab-76003, Abcam, Cambridge, MA), and VEGFR-3 (1:1000, sc-144000, Santa Cruz Biotechnology, Santa Cruz, CA), were diluted in 1% BSA in Tris-Buffered Saline Tween-20 (TBS-T) and incubated for 24 hours with the membranes on a shaker. Primary antibodies were washed three times for 10 minutes with TBS-T before adding anti-mouse (trypsin), anti-goat (chymotrypsin), or anti-rabbit (MMP-9, VEGFR-3) secondary antibodies (1:3000: Santa Cruz Biotechnology). Secondary antibodies were washed (3x, 10 min). Bands were measured using enhanced chemiluminescence pico (chymotrypsin, MMP-9) or femto (trypsin, VEGFR-3) substrates. Bands were quantified using digital densitometry.

F. MEASUREMENT OF LABEL DENSITY

Image processing was carried out using Image J (<http://rsbweb.nih.gov/ij/>).

G. STATISTICAL ANALYSIS

All measurements are presented as mean±standard deviation, with N=5 rats per group. Analysis of variance between groups was computed. Paired t-tests were used to compare protease activity measurements between time points and experimental groups for the casein digestion, charge changing substrates, and immunoblot measurements. P<0.05 was considered statistically significant.

III. Results

A. LYMPH FLOW RATE

The amount of lymph fluid collected for each time point varied between animals, with larger animals tending to produce more fluid (**Table 1**). During ischemia, there is a small quantity of lymph flow during the first 10-20 minutes, but it stops due to the lack of blood supply and interstitial fluid pressure in the splanchnic bed. To resume flow promptly at the start of reperfusion, it was sometimes necessary to gently press down on the intestine to enhance tissue fluid pressure. Severe dehydration was avoided by repeated intravenous booster shots (once every 20-30 minutes) each flushed into the animal with 0.1-0.2 ml of saline.

Table 1:

Lymph Volume collected during each time point for the shock and sham shock groups.

Experimental Group	Pre-Ischemia	Ischemia	Reperfusion Hour 1	Reperfusion Hour 2	Reperfusion Hour 3
SAO (ml)	0.73±0.20	0.14±0.06	6.80±0.83	7.05±0.62	6.90±0.34
SHAM (ml)	7.66±1.10	7.13±0.74	6.96±0.69	7.14±0.80	6.84±0.51

B. GENERAL PROTEASE ACTIVITY

Casein is a large globular protein that can be cleaved at several sites by multiple protease families. Therefore, it is useful as a marker for general protease activity. **Figure 2** shows a typical fluorescence curve for samples of postshock lymph and post sham-shock lymph (Reperfusion, Hour 3). All curves were standardized so that the

fluorescence at the first reading was assigned zero relative fluorescence units. This is reasonable since a time course of fluorescence changes is more indicative of activity than the starting point.

To analyze the fluorescence data and obtain a clear time course of protease activity, we measured the initial slope and the endpoint along the casein curves. The initial slope (**Figure 3**) represents the rate of casein digestion, which is larger with increased activity. Endpoints were calculated by averaging the last three data points on each curve (**Figure 4**), since they often stagger once the curve levels out. At this point, the proteases in lymph have cleaved all of the locations on casein that can be easily cleaved by those specific proteases (33). In theory, the curve would continue to increase very gradually until all locations on the molecule have been cleaved, though the limitations of fluorescent measurement do not allow us to accurately profile this activity. Both figures suggest that protease activity in lymph following splanchnic ischemia increases relative to sham shock lymph. They also suggest that the level of protease activity in control animals decreases during the five hours of surgery.

An unexpected finding of the study is that there is a base level of protease activity present in every animal. **Figure 5** shows the absolute fluorescent values of the slope and endpoint for the pre-ischemia time point of each group.

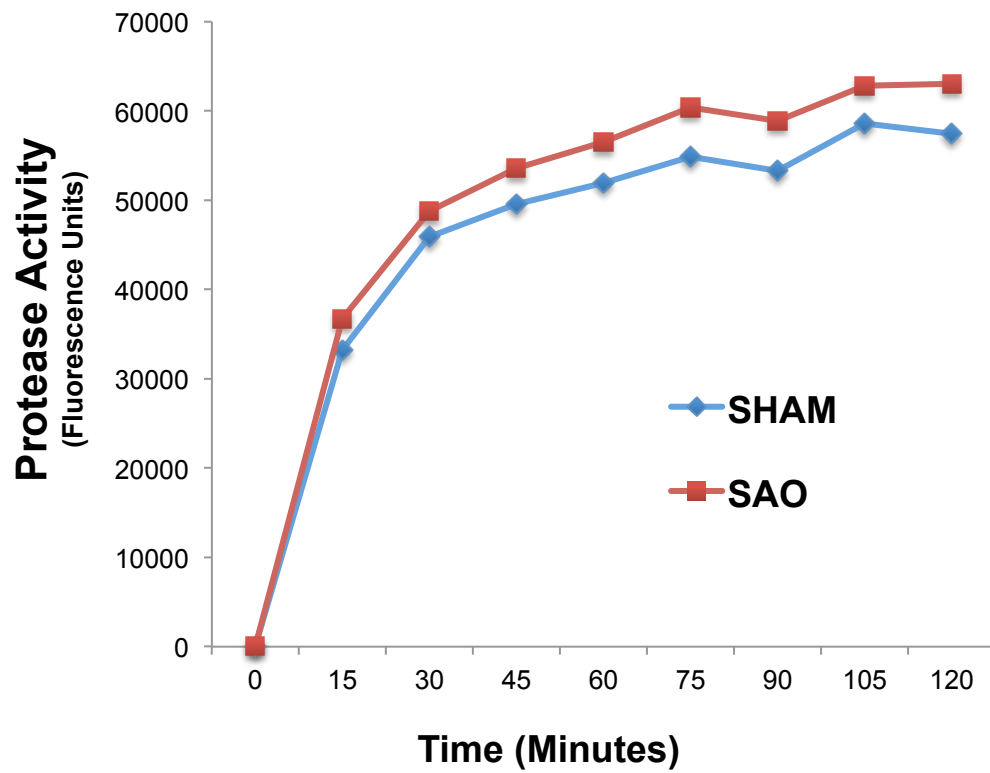


Figure 2: Fluorescence measurement of casein digestion by lymph fluid over time with readings taken every 15 minutes for 2 hours. SAO represents digestion from one sample taken during the third hour of reperfusion, while SHAM represents a sample from the corresponding sham shock time point.

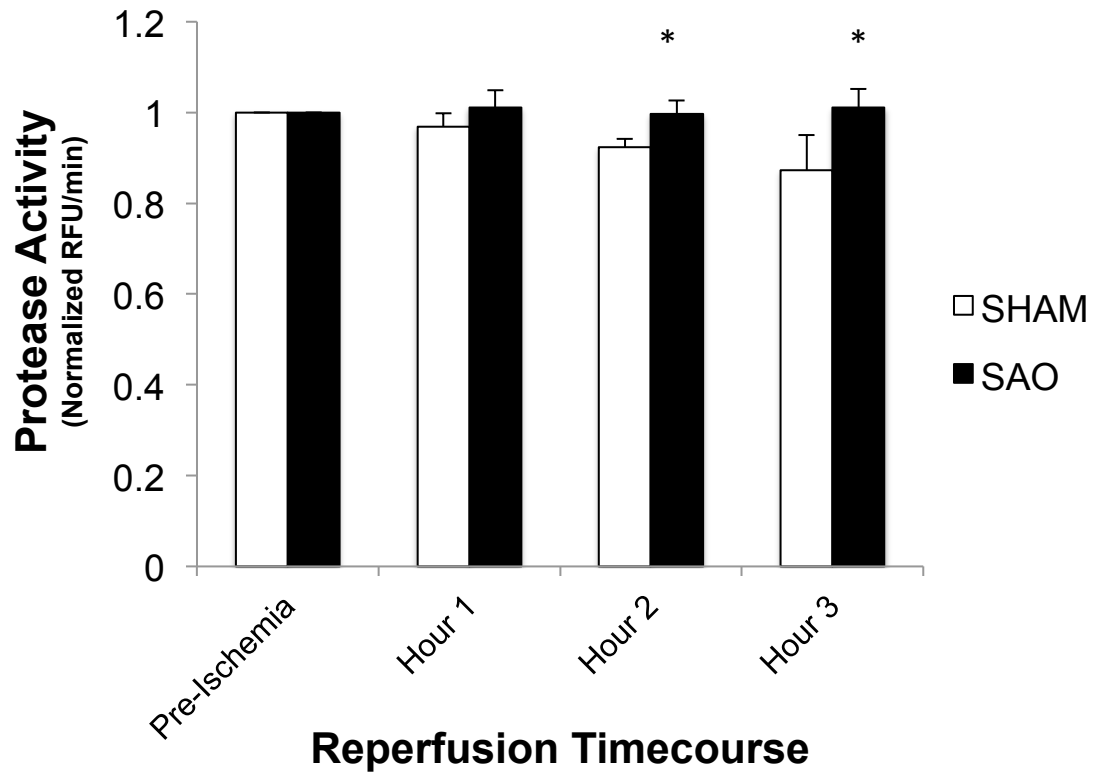


Figure 3: Protease activity of mesenteric lymphatic fluid as measured by the initial slope of casein digestion fluorescence curves. The differences in mean activity between the last two time points were statistically significant, $*p < 0.05$. $N=5$ for each group.

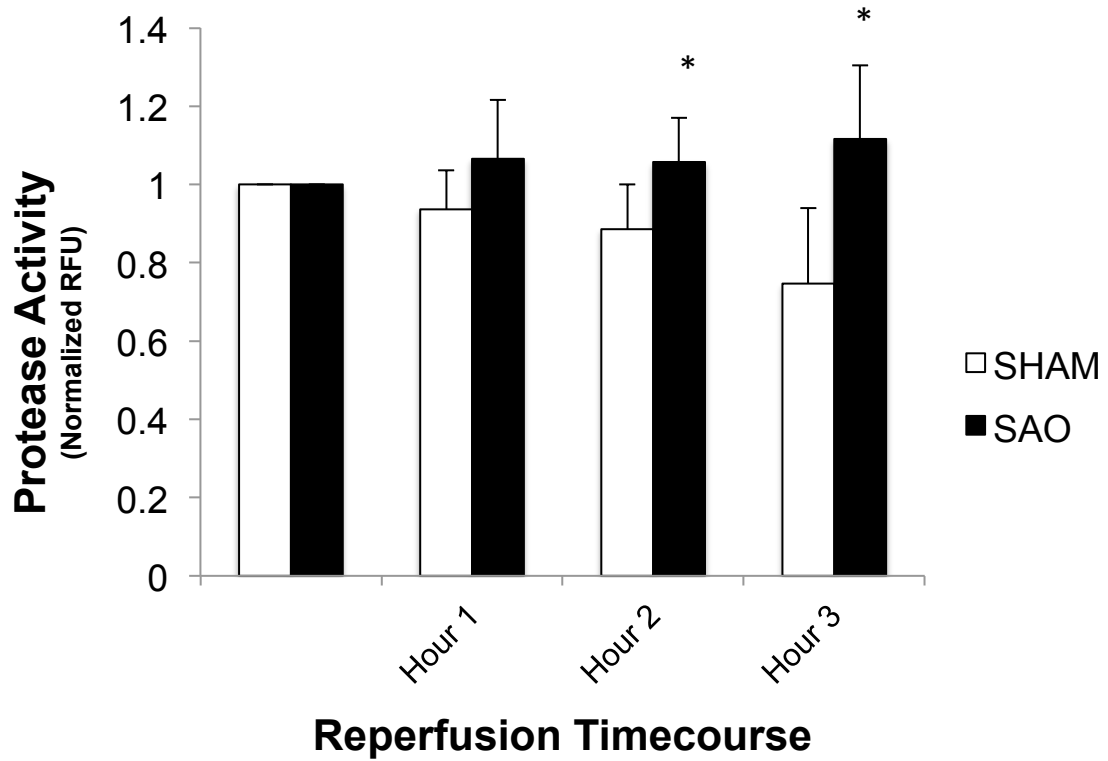


Figure 4: Protease activity of mesenteric lymphatic fluid as measured by the endpoint of casein digestion fluorescence curves. The difference in mean activity between the last two time points were statistically significant, * $p < 0.05$. $N=5$ for each group.

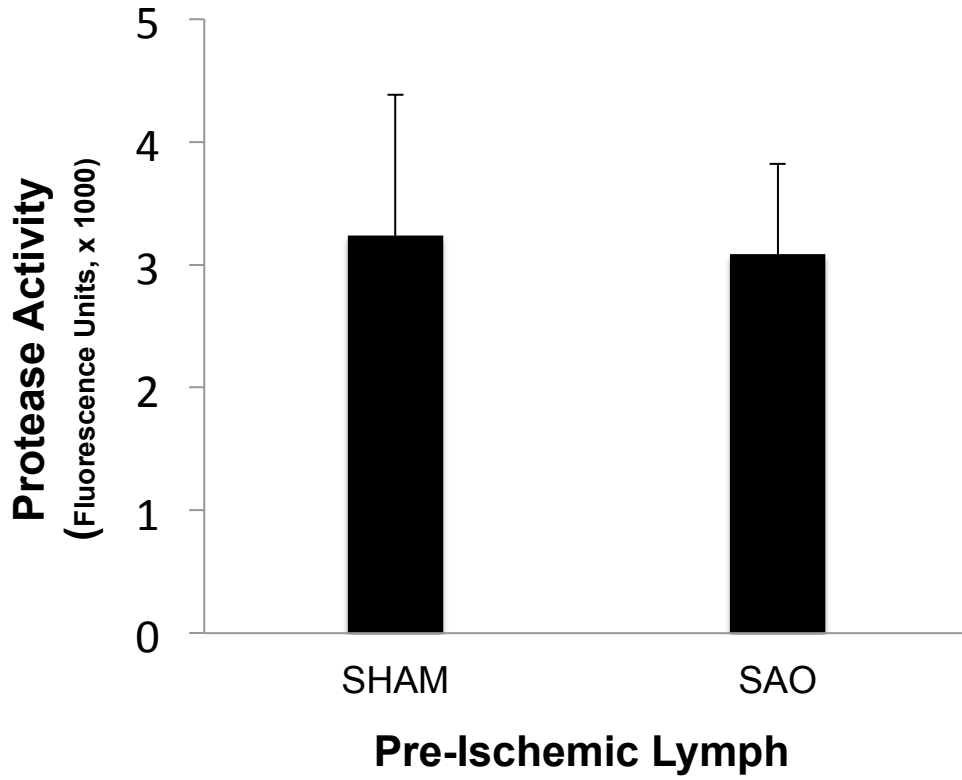


Figure 5: Protease activity of mesenteric lymph based on the endpoint of casein digestion after anesthesia and laparotomy. The difference between groups was not statistically significant. N=5 for each group.

C. SPECIFIC ENZYME ACTIVITY

Charge changing substrates were used to measure trypsin- and chymotrypsin-like activity in lymph. As with the casein measurements, the time course was normalized to pre-ischemia activity values, since the goal is to examine relative changes in concentration. In shock animals, trypsin-like activity during reperfusion was greater than during pre-ischemia, and remained elevated relative to the sham group through the three reperfusion time points (**Figure 6**). A similar trend was observed for chymotrypsin-like activity (**Figure 7**). Images of a typical gel for trypsin (**Figure 8**) and chymotrypsin (**Figure 9**) show the same qualitative trend.

Using preselected concentrations of exogenous trypsin and chymotrypsin, we obtained a calibration curve for each assay (**Figure 10**). The linear segment of each calibration curve allowed us to approximate the active enzyme concentration in each sample using a least squares fit. The mean trypsin and chymotrypsin activity is presented in **Table 2** and **Table 3** respectively. Note that these values are only approximate, since trypsin- and chymotrypsin-like enzymes do not cleave the substrates at rates equal to that of pure trypsin and chymotrypsin (32).

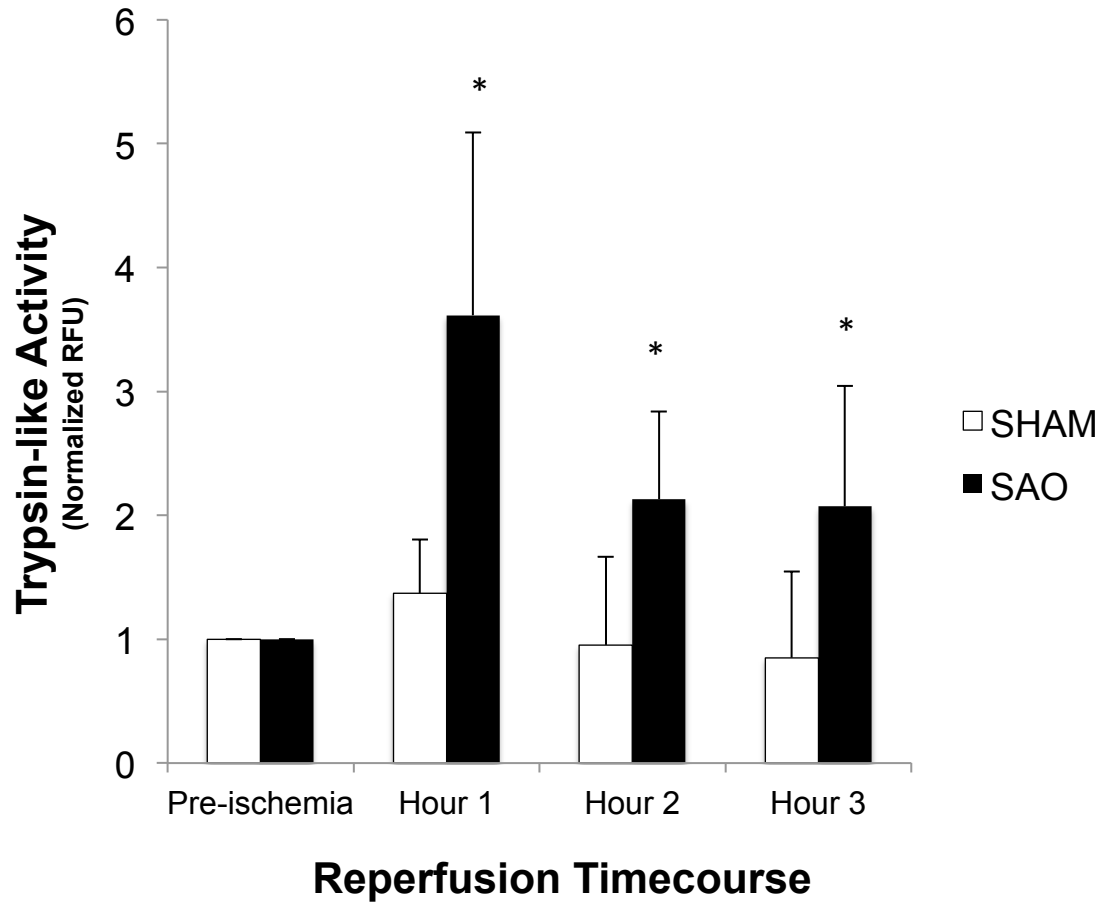


Figure 6: Trypsin-like activity in mesenteric lymph as measured by the mean fluorescence of a cleaved charge-separated substrate. The difference in mean activity between the last three time points were statistically significant, $*p < 0.05$. $N=5$ for each group.

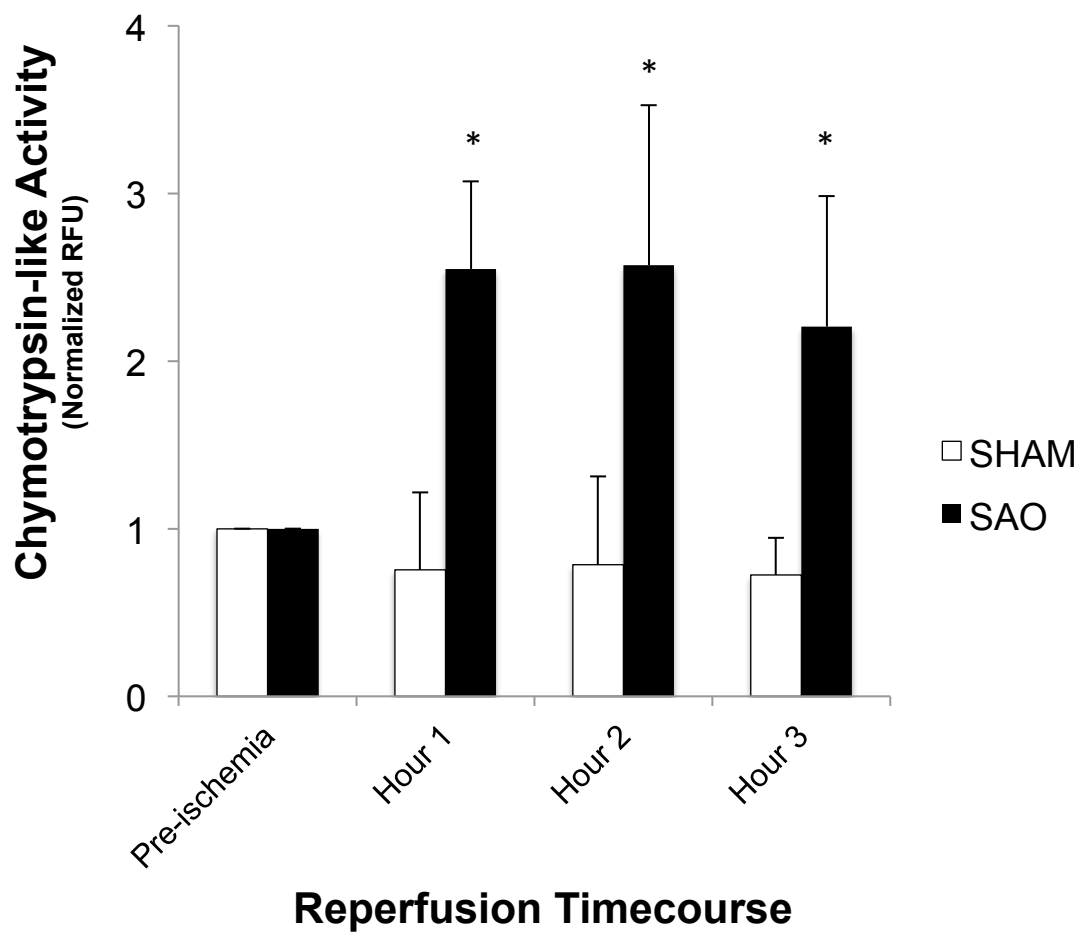


Figure 7: Chymotrypsin-like activity in mesenteric lymph as measured by the mean fluorescence of a cleaved charge-separated substrate. The difference in mean activity between the last three time points were statistically significant. * $p < 0.05$. $N=5$ for each group.

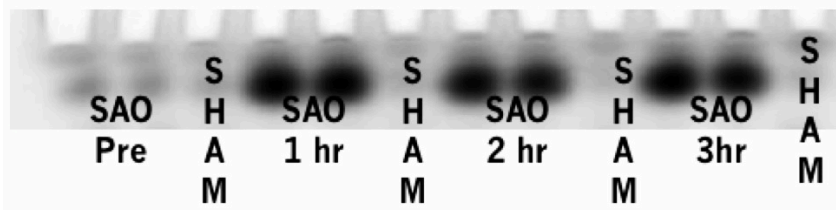


Figure 8: Photograph of a gel generated by a charge-changing substrate for trypsin after digestion by mesenteric lymph. Band density represents the extent of trypsin-like activity. Samples from the SAO group were run in duplicate. N=5 for each group.



Figure 9: Photograph of a gel generated by a charge-changing substrate for chymotrypsin after digestion by mesenteric lymph. Band density represents the extent of chymotrypsin-like activity. Samples from the SAO group were run in duplicate. N=5 for each group.

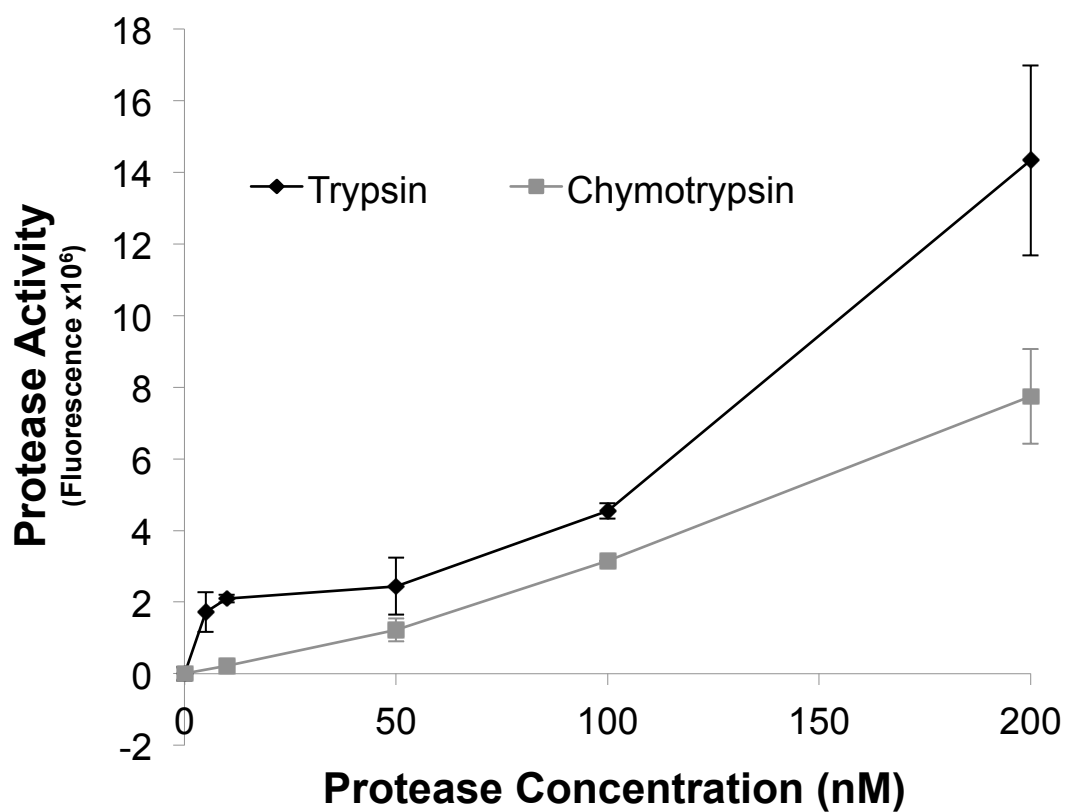


Figure 10: Calibration curve for charge changing substrates generated with selected concentrations of exogenous trypsin and chymotrypsin. N=3 for each enzyme.

Table 2:

Concentration of active trypsin-like enzymes in mesenteric lymph based on a calibration. N=5 for each time point.

Experimental Group	Pre-Ischemia	Reperfusion Hour 1	Reperfusion Hour 2	Reperfusion Hour 3
SAO (nM)	33.9±12.2	110.7±23.6	80.2±25.2	81.2±26.6
SHAM (nM)	38.7±9.5	42.8±11.5	32.1±10.2	27.9±7.9

Table 3:

Concentration of active chymotrypsin-like enzymes in mesenteric lymph based on a calibration. N=5 for each time point.

Experimental Group	Pre-Ischemia	Reperfusion Hour 1	Reperfusion Hour 2	Reperfusion Hour 3
SAO (nM)	18.2±1.6	31.8±3.8	31.3±4.5	30.6±5.7
SHAM (nM)	19.8±0.9	16.4±1.7	17.0±2.2	16.5±0.7

C. PRESENCE OF PANCREATIC PROTEASES AND MMP-9

Since charge-changing substrate detect only target trypsin- and chymotrypsin-like enzymes, it was important to confirm the presence of these pancreatic enzymes with immunoblotting. This technique allows for an approximation of relative protein concentration. We used primary antibodies against pancreatic trypsin (**Figure 11**), chymotrypsin (**Figure 12**), and MMP-9 (**Figure 13**). MMP-9 was included to test for presence of an additional protease other than those detected by charge changing substrates. For trypsin, bands were detected at the reported molecular weight, though they showed no significant change in concentration between groups or time points (**Figure 14**). The chymotrypsin antibody detected a protein at a molecular weight larger than that reported by the vendor, though again showing a constant concentration (**Figure 15**). MMP-9 was present at the reported molecular weight, though the concentration decreased through reperfusion (**Figure 16**).

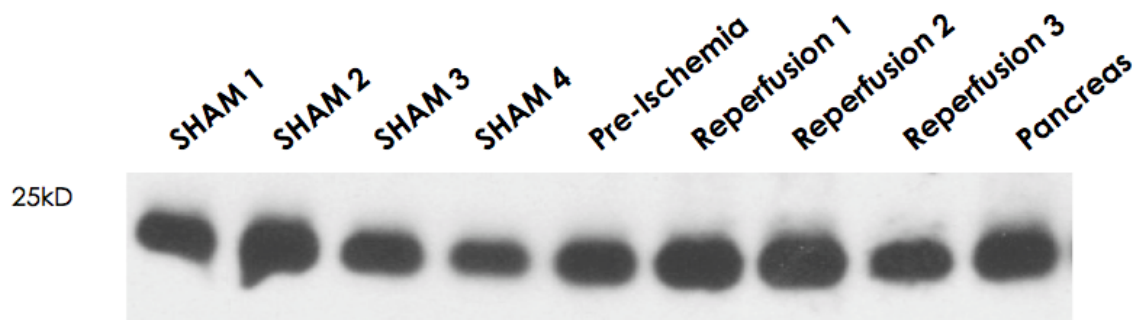


Figure 11: Westernblot for trypsin in mesenteric lymph revealing the 23kDa isoform.

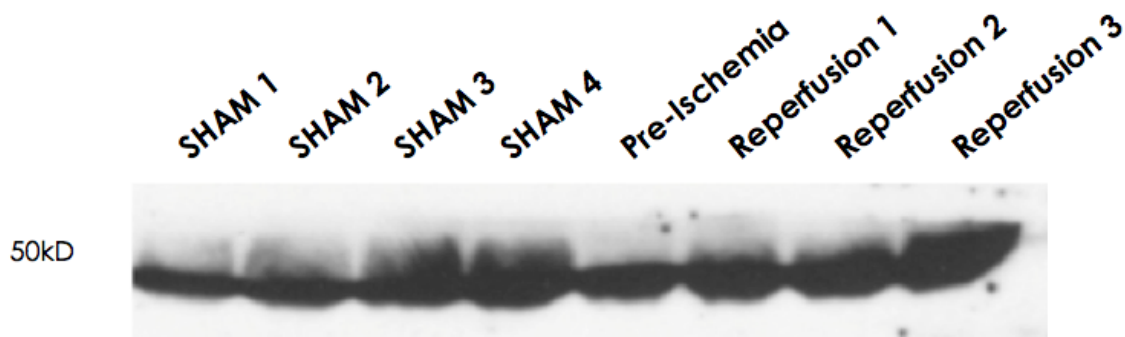


Figure 12: Westernblot for chymotrypsin in mesenteric lymph. No other bands were present.

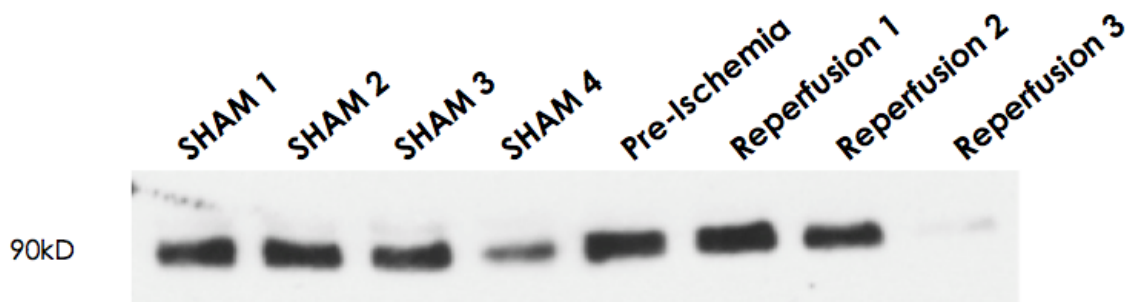


Figure 13: Westernblot for MMP-9 in mesenteric lymph revealing the 88kDa isoform.

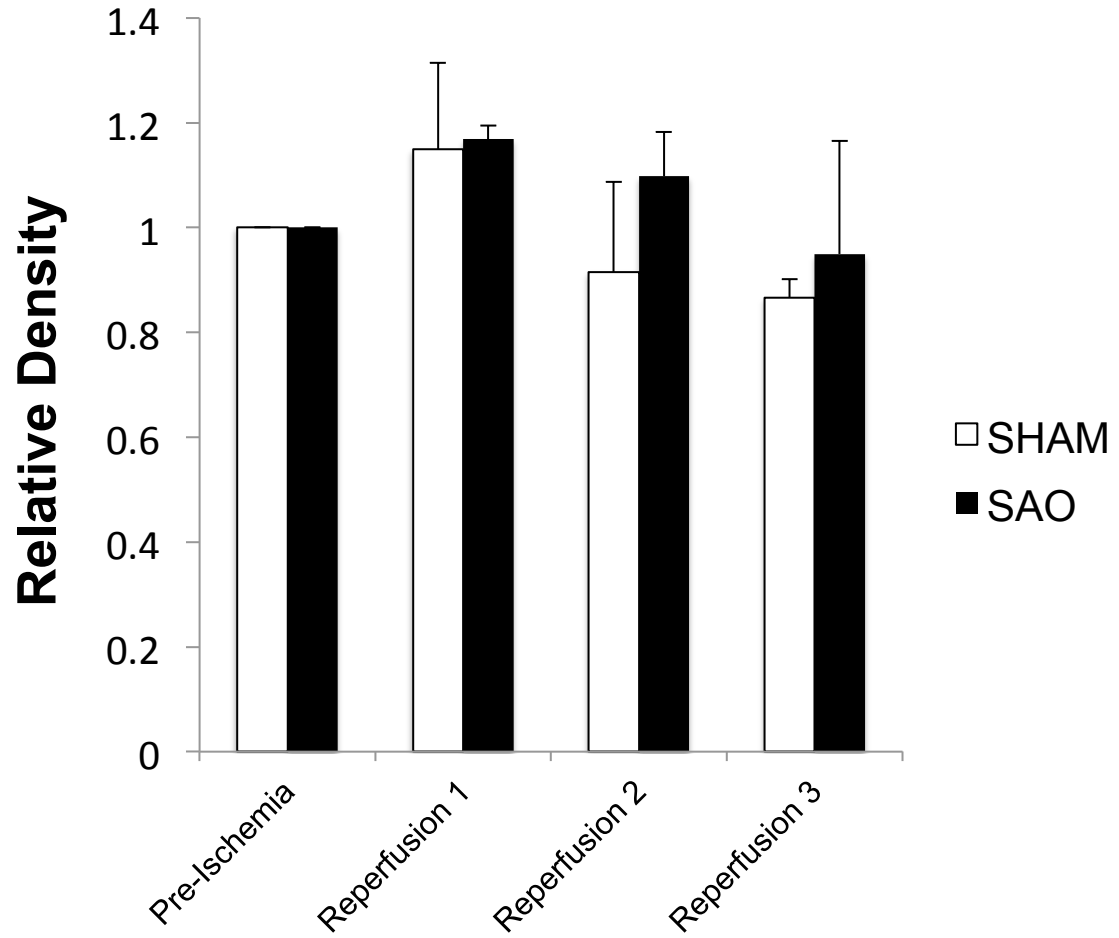


Figure 14: Densitometry for the trypsin Westernblot. The difference between groups was not statistically significant. N=3 for each group.

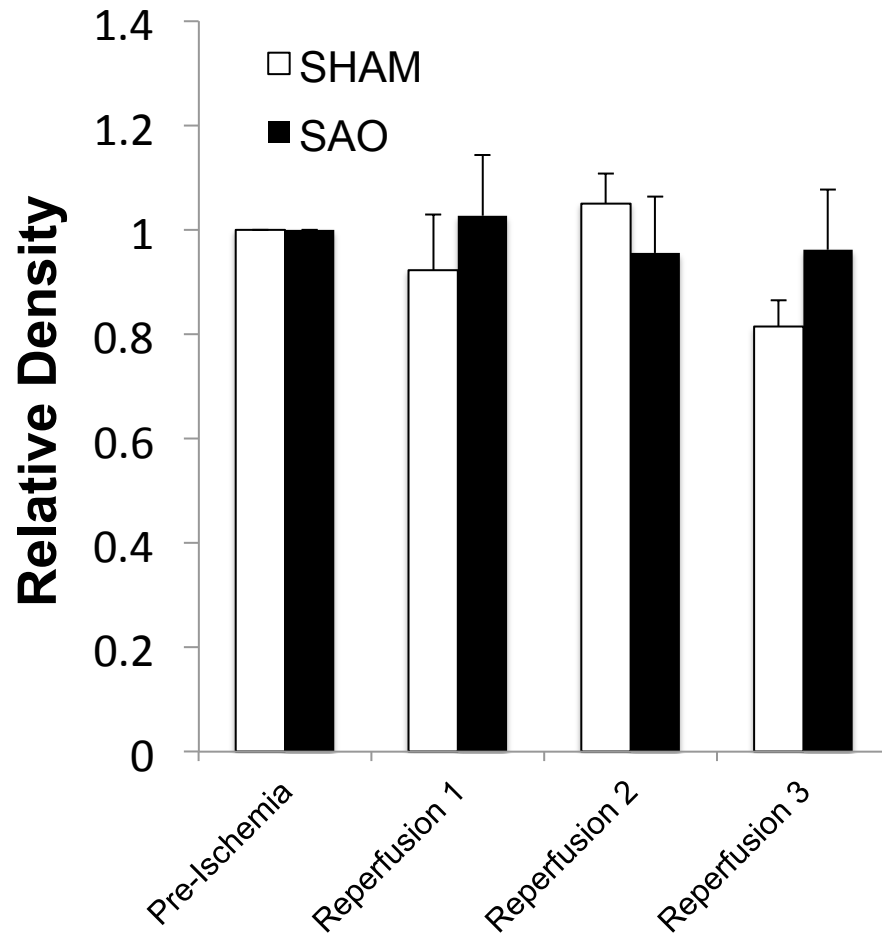


Figure 15: Densitometry for the chymotrypsin Westernblot. The difference between groups was not statistically significant. N=3 for each group.

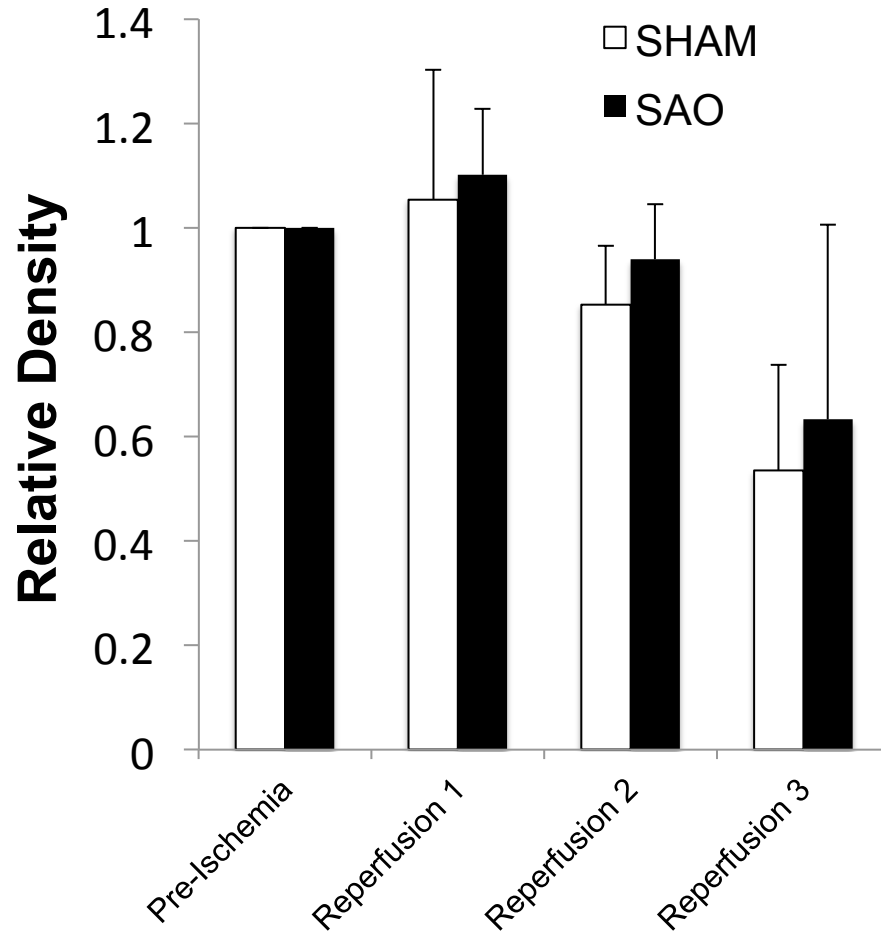


Figure 16: Densitometry for the MMP-9 Westernblot. The difference between groups was not statistically significant. N=3 for each group.

D. EVIDENCE FOR *IN-VIVO* PROTEASE ACTIVITY

Receptor cleavage can be used as a marker for unchecked protease activity. To determine whether the changes in artificial substrate cleavage correspond to an *in-vivo* phenomenon, we performed an immunoblot for VEGFR-3, an important lymphatic endothelial cell receptor (18). The blot (**Figure 17**) revealed a large number of bands, either corresponding to non-specific binding of the polyclonal antibody, or different sized fragments of the protein. The reported molecular weight is 170kDa for human VEGFR-3.

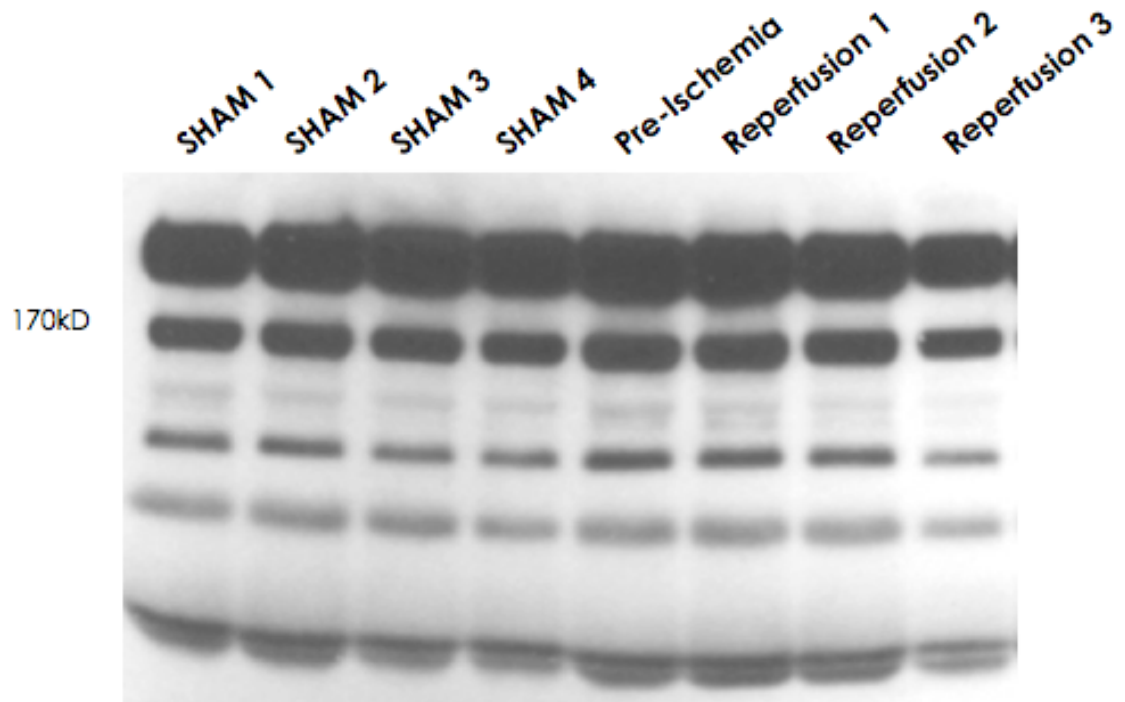


Figure 17: Westernblot for VEGFR-3 in mesenteric lymph.

IV. DISCUSSION

A. SUMMARY

There is a basic level of protease activity (in the nanomolar range) in mesenteric lymph that significantly increases relative to levels in sham animals after splanchnic arterial occlusion. General protease substrates and specific protease substrates for trypsin, chymotrypsin and MMP-9 reveal the enhanced activity after splanchnic arterial occlusion. Western analysis also revealed the presence of trypsin, chymotrypsin and MMP-9 in lymph. However, the differences in pancreatic enzyme protein concentration before and after shock were not significantly different, suggesting an increase in pro-enzyme activation.

B. PROTEASE ACTIVITY IN MESENTERIC LYMPH

The results of this study show clear evidence of increased protease activity in mesenteric lymph following intestinal ischemia. For both experimental groups, lymph fluid was collected for one hour preceding the hour of intestinal ischemia (or sham-ischemia). This sample provided a standard to compare the activity at later time points. Surprisingly, these samples displayed a significant amount of caseinolytic activity, with a mean endpoint of 30,288 fluorescent units, compared to the 11,013 fluorescent unit difference between the SAO and sham groups during the third hour of reperfusion. This suggests that there is a base level of unregulated proteases in lymph. There was a large variance in this activity between animals, with older animals tending to have greater proteolytic activity.

Relative caseinolytic activity increased throughout reperfusion. Since many different proteases are capable of cleaving casein, this can be interpreted as an increase in general proteolytic activity. However, there is only an increase in postshock activity when compared to sham animals. Casein digestion in the sham group decreased over the time course, with the SAO digestion remaining relatively constant. One possible explanation for this is the conditions of the surgical procedure. The rats were held without food or water for over six hours, and protease concentration in the intestine is known to change according to the needs of the digestive system. Without food, five hours of lymph collection may deplete the intestine's supply of proteases. It is also possible that dehydration or the effects of anesthetics play a role. Further, the immunoblot for MMP-9 showed a decrease in the enzyme's concentration over the time course of the experiment. This may provide a more mechanistic explanation for the decrease in casein digestion, since MMP-9 is capable of cleaving the protein (33). Whatever the cause, the reason for the decrease is most likely present in both groups, so the relative difference in activity can be attributed to the splanchnic occlusion.

Charge-changing substrates showed a more pronounced change in protease activity following shock. In the first hour of reperfusion, the trypsin- and chymotrypsin-like activity was two and three times greater than the normalized pre-ischemia levels, and remained elevated in the second and third hour. Since these assays are specific to pancreatic-like enzymes, it is likely that this activity originates in the intestine, rather than other mesenteric tissues or cells within the lymphatic system. The calibration curves also gave estimates for the concentration of active trypsin- and chymotrypsin-like enzymes in lymph. The 77 nM change in trypsin-like concentration is much smaller than

the reported 1–40 μM trypsin concentration in the intestinal lumen of rats, though it is larger than the 10 nM concentration in postshock plasma (28,34). The concentration may be quite significant since it is above the threshold for activation of the protease-activated receptor 2 (PAR-2), which modulates the inflammatory response to proteolytic activity (34).

Since enzymes other than trypsin and chymotrypsin can cleave charge-changing substrates, we confirmed the presence of these enzymes with Western analysis. While trypsin was detected in every lymph sample, there were no statistically significant changes in concentration over the time course of the current experiments. This does not rule out small changes in concentration that are beyond the detection of immunoblots, though it suggests that the two to three fold increase in trypsin- and chymotrypsin-like activity cannot be explained by concentration changes alone. One explanation for the situation is the decrease in inhibitor concentration documented in proteomic studies. Alpha-2-Macroglobulin and alpha-1-Inhibitor 3, both inhibitors of pancreatic enzymes, experience a -2.7 and -2.1 fold change respectively in the second hour of reperfusion compared to pre-ischemia in a hemorrhagic shock model (21). This decrease is approximately equal to the increase in activity measured by charge-changing substrates. A second explanation may be that enzymes other than trypsin and chymotrypsin are present in quantities large enough to cleave the substrates. Pancreatic amylase has been detected in postshock lymph through proteomics and could contribute to the digestion our substrates (22).

Even with the uncertainties associated with the enzyme identity, concentration, and inhibition, the finding that general and pancreatic-like activity increases is

pathophysiologically significant. The concentration of active enzymes was high enough to activate PAR-2, and there is already evidence that pancreatic proteases can activate MMPs (28), suggesting that the systemic inflammation that accompanies MOF may result from protease activity. These findings suggest a possible mechanistic starting point for some of the most clinically relevant consequences of circulatory shock, and highlight limitations of proteomic studies when looking at enzyme cascades.

C. GUT BARRIER DYSFUNCTION

While these results suggest that proteases can contribute to the pathogenesis of inflammation and MOF after splanchnic IR, they do not provide conclusive evidence for or against the intestinal permeability proposed by the *Autodigestion Hypothesis*. The hypothesis is that proteases in the lumen of the intestine escape as a result of a damaged mucosal barrier (6). Since the lymph is the conduit for intestinally absorbed molecules, it is possible for the lymphatics to carry these proteases into the circulation. However, the mesenteric lymph duct is the collecting point for the entire splanchnic bed, including the pancreas and liver, so the trypsin and chymotrypsin we detected in this fluid could come from sources other than just the intestine (35).

While attributing mesenteric lymph solely to the intestine is common throughout the shock literature (9, 11, 13), it has no bearing on the theory of Autodigestion. Studies that do not use the lymph duct as a sample collection point show that proteases escape the intestine. Trypsin, chymotrypsin, and elastase concentrations are elevated in homogenates of the intestinal wall, mesentery, and peritoneal fluid (23, 27, 28). The only reason I chose to look in lymph was to see if this elevation is related to the MOF inducing effects

of postshock lymph. These results may not give evidence for the Autodigestion theory, but there is certainly a basis for implicating the intestine as a likely source of proteases.

D. EXPERIMENTAL LIMITATIONS

A main limitation in this study is the inability to determine whether the proteases in mesenteric lymph are flowing out from the intestine or the pancreas. However, the intestine is a more likely source. In study of rats with acute pancreatitis, mesenteric lymph contained amylase, trypsinogen, and chymotrypsinogen, but not trypsin or chymotrypsin (36). While this is a different form of injury from IR, it is further evidence that the zymogens of these enzymes predominate in the pancreas, and it is therefore more likely that any trypsin in the lymph came from the intestine. The trypsin antibody we used for our immunoblot was monoclonal, and the vendor reported that it is able to distinguish the enzyme from its precursor.

The use of anesthetics is another limitation. Due to restrictions in the availability of sodium pentobarbital, we used a combination of ketamine and xylazine, both of which have effects that could influence the shock symptoms. Ketamine is known to delay mortality and the subsequent sepsis in a hemorrhagic shock model (37), and xylazine can cause myocardial depression and bradycardia, which was observed in the blood pressure traces. This limits the quantitative accuracy of our results and the extent of damage after IR, but there is no reason to suggest it would interfere with the actual mechanisms and pathogenesis of postshock inflammation and organ failure.

Finally, our methods of detecting protease activity are only *in-vitro* approximations. Casein and the charge-changing substrates are meant to simulate what

may happen if the lymph were to enter the rat's circulation, but we cannot be certain. The attempt to look at whether the lymphatic endothelial cell receptor (VEGFR-3) was cleaved following shock was limited by the non-specific binding of the polyclonal antibody, and the possibility that the receptor is further degraded in the lymph fluid.

E. CONCLUSIONS

This study represents the first effort to profile protease activity in mesenteric lymph. The general protease activity increases after splanchnic arterial occlusion. Trypsin- and chymotrypsin-like activity also increases, although there is no evidence of large concentration changes for these enzymes. This increased activity implicates proteases as a possible mediator for the systemic inflammation and subsequent organ failure seen in patients suffering from circulatory shock.

V. FUTURE STUDIES

The method we developed for collecting lymphatic fluid could be useful in further studies on circulatory shock. Free fatty acids have been identified as potential cytotoxic mediators in shock, and it would be valuable to determine their concentration just as we did with protease activity. Various forms of albumin have also been shown to activate neutrophils and trigger the inflammatory cascade, and could easily be investigated with similar methods (3).

The protease activity outlined in this study also demands further investigation. It is reported that pancreatic protease activity can activate MMPs, which then go on to cleave cell receptors in the lung, but this needs to be investigated directly. Postshock lymph fluid could be tested for its ability to activate MMPs, and could even be injected into a naïve rat to measure its ability to cleave receptors other than VEGFR-3.

While the idea that the intestine is involved in the pathogenesis of shock is widely accepted (5), there are few experiments to determine whether it is the sole source of the molecules causing multiple organ failure. It is well documented that the lymph flowing from the splanchnic bed can induce lung damage, cell cytotoxicity, etc., but it is unclear whether it is unique in this ability. It is possible that many different types of tissues generate cytotoxic mediators after ischemia and reperfusion, and the shock research is simply focused on a large tissue that happens to produce a high percentage of the body's total lymph fluid.

A simple experiment could potentially address this issue. Mesenteric lymph collected from a rat undergoing hemorrhagic shock could be delivered to a tethered naïve

animal, as done in a previous study (16). Then, the organ damage induced in the naïve animal by the transfused lymph fluid should be compared to that of an animal subjected to the same shock model, but with no interference with lymph flow. Stringent controls for the animal age, weight, and diet would have to be set. If the organ damage is the same in both groups, then it is clear that the splanchnic bed is the sole source of cytotoxic mediators. This would give important insight into the mechanisms of MOF, and have implications for potential treatments of circulatory shock.

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