RESEARCH PLAN

Specific Aims

Trauma is a major cause of death and disability worldwide. Hemorrhagic shock is a significant etiology of these deaths. Standard resuscitation strategies, such as those promoted by the American College of Surgeons' Advanced Trauma Life Support course (1) emphasize early resuscitation with crystalloid fluids, followed by red blood cell transfusion for non-responders. When patients have significant hemorrhage, this strategy may be associated with the onset of coagulopathy, which may in turn result in continuing exsanguination.

Upon surviving an initial insult, patients are placed at risk for developing the systemic inflammatory response syndrome (SIRS), which may progress to multiple organ dysfunction syndrome (MODS), the most common cause of late death following trauma (2). SIRS is a clinical syndrome of dysfunctional acute inflammation in which the inflammatory response becomes maladaptive and leads to organ damage. A hallmark of the acute inflammatory response is activation of pro-inflammatory transcription factors leading to increased production of pro-inflammatory cytokines, and upregulation of cellular adhesion molecules. These events lead to organ infiltration of neutrophils with resultant organ injury and dysfunction.

Recent innovations in clinical resuscitation strategies have been proposed as an attempt to blunt the coagulopathy associated with acute hemorrhage. These clinical protocols represent a significant paradigm shift from traditional care and involve liberal use of packed red blood cells and plasma as resuscitation fluid, as well as minimization of crystalloid infusion. This new strategy, termed "damage control resuscitation" appears to reduce immediate deaths secondary to massive transfusion in both military and civilian populations (3, 4). Initial clinical retrospective studies have suggested that survival is increased with transfusion of increasing ratios of plasma to red blood cells, but the optimal ratio of blood component utilization in damage control resuscitation as well as the effect of this new strategy on the inflammatory response to hemorrhage remain unknown.

Decreasing death from coagulopathy is a substantial advance in the care of acutely injured patients. However, initial survival from the coagulopathy of hemorrhagic shock leaves a significant number of patients at risk of developing dysfunctional inflammatory responses and SIRS, which may lead to multiple organ failure and death. To date, there is no data to delineate the effect of damage control resuscitation strategies on the inflammatory response to hemorrhagic shock and ensuing end organ damage from SIRS.

The global hypothesis of the proposed study is that damage control resuscitation is superior to standard resuscitation by blunting the acute inflammatory response and onset of remote organ injury. An additional goal is to determine optimal ratios of plasma to red blood cells for use in damage control resuscitation. To investigate this, we propose three specific hypotheses:

1. Damage control resuscitation strategies lead to increased survival and decreased organ injury after hemorrhage. To test this, we will determine the effect of resuscitation with crystalloid fluids, fresh whole blood, or different ratios of plasma to red blood cells after hemorrhage on:

a. Mortality after hemorrhage.
b. Function and architecture of the intestine, liver, lung, and kidney.

Results of this aim will determine the optimal ratio(s) of plasma to red blood cells to be used in subsequent aims.

2. Damage control resuscitation strategies are associated with diminished pro-inflammatory transcription factor activation and mediator production after hemorrhage. To test this, we will determine the effect of hemorrhage and resuscitation with the optimal ratio of plasma to red blood cells as compared to conventional crystalloid resuscitation and fresh whole blood on:

a. Activation of the pro-inflammatory transcription factors NF-κB and AP-1 in intestine, liver, lung, and kidney.
b. Organ specific mRNA expression of pro-inflammatory cytokines and vascular cell adhesion molecules.
c. Systemic and organ-specific production of pro-inflammatory cytokines and upregulation of vascular cell adhesion molecules.

These studies will identify organ-specific effects of resuscitation strategies on transcription factors and mediators that regulate the systemic inflammatory response.

3. Damage control resuscitation strategies alter neutrophil function after hemorrhage. To test this, we will determine the effect of hemorrhage and resuscitation with the optimal ratio of plasma to red blood cells as compared to conventional crystalloid resuscitation and whole blood on:

a. Neutrophil infiltration and redistribution in lung, liver, intestine, and kidney.
b. Systemic neutrophil activation and function.

These studies will determine the effect of resuscitation strategies on neutrophil function, activation and trafficking.

Together, the proposed experiments will increase our understanding of the effect of damage control resuscitation on post-injury inflammation and organ damage as well as provide information on the effect of different ratios of plasma to blood during resuscitation from hemorrhagic shock.

Background and Significance

Hemorrhagic shock and resuscitation. Trauma is a major cause of death, accounting for up to 150,000 deaths per year in the United States (5). Injuries are the leading cause of death for people from age 1 to 44 in the United States (5) and from age 5-44 world wide (2). Of deaths from trauma, nearly 80% occur within 48 hours of injury (5), with hemorrhage accounting for 30-40% of all trauma fatalities (6). Improvements in trauma care and emergency medical services have resulted in decreases in potentially preventable deaths, but trauma remains a deadly disease.

In caring for the traumatized patient, initial hemorrhage control is essential. Failure to achieve this is a major cause of preventable trauma deaths, accounting for at least 25% of patients in this category (7). If patients survive their initial insult, they are at risk for the onset of the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), the leading cause of late death from trauma (2, 5). While potentially preventable deaths may occur secondary to hemorrhage immediately post-injury, multiple organ failure is a major cause of death later in the post-injury course. Understandably, the resuscitation of traumatically injured patients continues to be the focus of intensive investigation.

For more than 30 years, initial resuscitation of patients has been guided by the American College of Surgeons' Advanced Trauma Life Support course (1). Initial resuscitation is predicated on

expanding intravascular volume as well as replenishing extravascular losses, followed by transfusion of packed red blood cells (pRBCs) for ongoing hypotension. If coagulopathy develops at a later time, coagulation factors are replaced with transfusion of fresh frozen plasma (FFP), cryoprecipitate, or platelets, based on laboratory studies of coagulation factors. For example, current clinical standard of care is to transfuse one unit of FFP for every four to ten units of pRBCs in the event that either the prothrombin time is elevated, indicating coagulopathy, or a total of ten units pRBCs have been transfused (8). ***Ongoing initiatives in clinical care are targeted towards quickly and precisely resuscitating patients from hemorrhagic shock, but the optimal resuscitation strategy as well as its effect on subsequent patient recovery remains unknown.***

Hemorrhagic shock, SIRS, and MODS. Hemorrhagic shock and resuscitation induce a whole-body ischemia/reperfusion state that may result in SIRS. SIRS is characterized clinically by tachycardia, tachypnea, elevated or decreased body temperature, or increased or decreased white blood cell count (9). SIRS may progress to MODS, which is typified by altered function of vital organs, especially lungs, liver, kidney, and intestine. MODS is the most common cause of late death from trauma.

The pathophysiology of SIRS and MODS is complex. SIRS represents a state of dysfunctional inflammation and is associated with elevated production of proinflammatory cytokines such as TNF-α, IL1β, and IL-6 (reviewed in 10). Each of these is a major regulator of the inflammatory response and stimulates production of additional downstream mediators, including chemokines and adhesion molecules (11). Together, these proteins initiate chemotaxis and migration of neutrophils into tissue. The infiltration of activated neutrophils in organs is associated with increased organ dysfunction and tissue injury (10, 11).

Substantial data suggest that hemorrhage induces a pro-inflammatory state that may result in SIRS and MODS. One series of experiments in a hemorrhagic shock and resuscitation model found increased intestinal levels of the pro-inflammatory proteins IL-6, iNOS, ICAM-1, and G-CSF after hemorrhage (12). The chemokines MCP-1 and MIP-1α were shown to be important in the evolution of inflammation and injury following hemorrhage (13, 14). In another study, hemorrhagic shock led to increased hepatic and ileal IL-6 and TNF-α mRNA levels (15). Additional studies strongly suggest that IL-6 is important in the development of intestinal barrier failure after hemorrhagic shock in mice (16). A recent series of experiments found that mesenteric levels of TNF-α and IL-6 were increased by hemorrhagic shock and resuscitation (17). Of note, mesenteric levels of IL-6 after resuscitation were increased beyond systemic levels, suggesting that intestine is an important source of this cytokine under these conditions (17). Together, these studies indicate that the pro-inflammatory state occurring after hemorrhage is an important component of the pathogenesis of SIRS and MODS.

Many of the pro-inflammatory changes that occur after hemorrhagic shock and resuscitation appear to be controlled by the transcription factors nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1). NF-κB regulates a large number of genes for many proinflammatory cytokines and acute phase proteins. First described more than 20 years ago by Sen and Baltimore (18) as a nuclear factor responsible for immunoglobulin light chain transcription in B cells, NF-κB was subsequently found to be present in non-immune cells and tissues, including lung, intestine, kidney, and liver. Additional work discovered that NF-κB activation occurs under various conditions, particularly acute inflammation. Due to its central role in the inflammatory process, NF-κB continues to be the focus of intense investigation.

Under basal conditions, NF-κB is normally sequestered in the cytoplasm by the inhibitory protein IκB. In response to a stimulus, activation of a series of kinases leads to the phosphorylation of IκB (most commonly IκB-α), leading to poly-ubiquitination and degradation by the proteasome. IκB-α degradation exposes a nuclear localization sequence on the NF-κB complex, leading to nuclear translocation, binding to target sequences, and transcription of target genes, including IL-1β, TNF-α,

IL-6, and IL-8 (reviewed in 19). NF-κB activation is usually rapid, transient, and self-limited. NF-κB also induces transcription of several IκB proteins, which terminate NF-κB activation by re-sequestering NF-κB in the cytoplasm.

AP-1 is a dimer composed of members of the jun (jun-B, c-jun, and jun-D) and Fos (c-fos, fos-B, fra 1, and fra 2) families. AP-1 activation may be initiated by cellular stress or pro-inflammatory cytokines, leading to increased transcription of AP-1 proteins, binding to target DNA sequences, and altered transcription of target genes, including TNF-α, IL-1β, IL-6, and the chemokine IL-8 (20).

Due to the key role that NF-κB and AP-1 likely play in the response to acute inflammation, investigators have examined its role in the several tissues, including liver, kidney, lung, and enterocyte and intestinal mucosa (reviewed in 20). NF-κB or AP-1 activation in one tissue may have effects in other organs as well. For example, inhibition of NF-κB activation in a model of superior mesenteric artery occlusion diminished subsequent tissue injury as well as TNF-α production locally in intestine as well as remotely in lung (21). Initial studies have begun to examine the role of NF-κB and AP-1 in the acute inflammatory response to hemorrhagic shock. One series of experiments demonstrated NF-κB activation after hemorrhagic shock in liver, kidney, and intestine. This was inhibited by the non-specific NF-κB inhibitors DMSO, PDTC, methylprednisolone (22). Additional experiments suggest that AP-1 and NF-κB activation occur in liver following induction of hemorrhagic shock (23) and that inhibition of activation of these transcription factors with curcumin is associated with decreased hepatic injury (24). Together, these studies suggest that NF-κB and/or AP-1 are central regulators of the inflammatory response to hemorrhagic shock.

Another key component in the pathogenesis of SIRS and MODS is the neutrophil. Circulating neutrophils respond to cytokines and chemokines released during injury and inflammation. This initiates a series of events leading to neutrophil emigration from the vasculature into tissues, including lung, liver, kidney, and small intestine. Several components of neutrophil migration and activation are regulated by chemokines such as IL-8. Subsequent release of superoxide anions and proteolytic enzymes may directly injure cells, leading to organ dysfunction (reviewed in 25). As the cellular "first responder", inappropriate activation and recruitment of neutrophils, as occurs during hemorrhagic shock, represents a critical component in the development of MODS.

Resuscitation strategies for hemorrhage. Several resuscitation strategies have been employed clinically and experimentally in an attempt to decrease morbidity and mortality after hemorrhage. Crystalloid resuscitation, with solutions such as Ringer's lactate, continues to be the mainstay of clinical practice. In the event of ongoing blood loss, crystalloid fluids are supplemented with blood products to attempt to correct anemia or coagulopathy. Experimental data indicate that components of Ringer's lactate induce a pro-inflammatory state and contribute to end organ damage (26). In another study, male rats were subjected to hemorrhagic shock and resuscitated with either shed blood, half shed blood and half shed blood volume given as Ringer's lactate solution, or 3 times the shed blood volume as Ringer's lactate. While each group had increased evidence of intestinal injury and lung injury, gut injury was worse in the group resuscitated with Ringer's lactate than in the other groups (27). Together, these studies suggest that although it is considered the standard of care, crystalloid based resuscitation appears to induce an inflammatory response and may contribute to the pathogenesis of SIRS and MODS.

Investigators have compared the use of crystalloid fluid with colloid fluids after injury. In an animal model of moderate splenic injury, the hemodynamic response to crystalloid or colloid resuscitation was similar (28). An elegant study of endothelial cell dysfunction in hemorrhagic shock suggested that crystalloid solutions containing blood are associated with less microvascular dysfunction than those without (29). Another series of experiments compared the use of shed whole blood, Ringer's lactate and hydroxyethyl starch or Dextran 40. The investigators found decreased renal damage with shed blood or Dextran 40 as compared to the other groups (30). Based on this

data, it appears many resuscitation strategies utilizing crystalloid fluid may be associated with increased organ injury. Although colloid fluids may be superior to crystalloid solutions in some aspects, large scale clinical trials do not support their current use as the superior fluid for resuscitation from hypovolemic shock (31).

Several pre-clinical studies have examined the use of modified crystalloid and colloid fluids for resuscitation after hemorrhage. One series of experiments suggested that resuscitation with a solution of hypertonic saline and the phosphodiesterase inhibitor pentoxifylline was associated with decreased lung and gut injury as compared to controls 24 hours after hemorrhage (32). It appears that use of hypertonic saline solution with pentoxifylline as compared to Ringer's lactate is associated with decreased hepatic levels of NF-κB, iNOS, HMGB1, and STAT3 as well as decreased intestinal NF-κB, TNF-α, IL-6, iNOS, and CINC (33, 34). An additional report found that resuscitation with hypertonic fluids prevented post-resuscitation vasoconstriction and improved endothelial cell function (35). Resuscitation with hydroxyethyl starch solution decreased NF-κB activation in liver and lung as compared to Ringer's lactate solution (36). Use of hypertonic saline solution resulted in decreased neutrophil activation than did resuscitation with Ringer's lactate (37). Hypertonic saline resuscitation, as compared to normal saline was associated with less gut neutrophil infiltration and mucosal injury (38). Although these modified resuscitation fluids are not employed clinically, these reports strongly suggest that specific resuscitation strategies in hemorrhagic shock have disparate effects on organ damage, hemodynamic response, transcription factor activation, cytokine production, and neutrophil activation. Thus, resuscitation strategies, in and of themselves, offer an opportunity to therapeutically modify the inflammatory response to hemorrhage, with the possibility of blunting SIRS and MODS.

Damage control resuscitation. Recently, standard clinical resuscitation practice for hemorrhagic shock has been challenged. Clinical data from a civilian trauma database suggested that standard resuscitation protocols do not adequately correct coagulopathy. These authors proposed earlier and more aggressive fresh frozen plasma (FFP) transfusion (8). An early analysis of cause of death in Operation Iraqi Freedom and Operation Enduring Freedom indicated that hemorrhage was the most common cause of potentially survivable deaths in these conflicts (39). Together, this information led to the hypothesis that increased transfusion of blood and FFP, as compared to standard resuscitation practice, could be beneficial to patients.

Termed "damage control resuscitation," this strategy departs from ATLS guidelines and is characterized by minimized therapy with crystalloid fluids and early use of fresh whole blood or FFP and pRBCs in ratios as high as one unit of FFP per each unit of pRBCs. In one retrospective review, early use of fresh whole blood was noted to be associated with increased survival in military trauma patients as compared to pRBCs (40), but fresh whole blood is not readily available for use in trauma resuscitations, so clinicians have turned to component therapy, predominantly with pRBCs and FFP, to attempt to decrease mortality. Recent clinical data from a military setting suggests that massively transfused patients who received a higher ratio of plasma to pRBCs had a significantly lower mortality rate, at least in part due to decreased death from hemorrhage (3). In this study, mortality in the high plasma to pRBC group of patients was 19%, while mortality in the low plasma to pRBC group was 65% (3). In civilian practice, more aggressive use of FFP was also associated with decreased mortality as well as decreased overall blood product use (41). The initiation of damage control resuscitation has modified clinical practice in many trauma centers, leading to more liberal transfusion of FFP and pRBCs as initial trauma resuscitation. Early data continues to be retrospective in nature and suggests that a ratio of one unit FFP to each unit of pRBCs was associated with increased survival (42). An additional study indicated that an FFP to pRBC ratio greater than 2:3 was an independent predictor of decreased 30 day mortality (43). Delineation of guidelines for optimal FFP and pRBC utilization in damage control resuscitation await prospective trials. ***Despite promising clinical data, the ideal ratio of FFP to pRBC transfusion in damage control resuscitation is unknown.***

Adoption of damage control resuscitation strategies in resuscitation from hemorrhage requires increased amounts of FFP and pRBCs as compared to standard therapy. Transfusion of blood and blood products is not without risk. A recent systematic review of the literature suggests that risk associated with pRBC transfusion outweighs potential benefits in critically ill patients (44). Blood and blood component therapy in trauma patients is associated with increased rates of infection, MODS, SIRS, and the adult respiratory distress syndrome (44). This is consistent with data from early studies of damage control resuscitation. In one study, death secondary to sepsis and MODS occurred in 19% and 13%, respectively, of patients receiving high ratios of FFP to pRBCs. As the ratio of FFP to pRBCs increased, so did the number of patients who eventually succumbed to SIRS related events, sepsis, and MODS (3), suggesting that survival from the initial insult may be associated with subsequent onset of SIRS and its sequelae. There is a paucity of data concerning the effect of damage control resuscitation, as compared to other resuscitation strategies, on the onset of organ dysfunction; production of pro-inflammatory transcription factors, activation of transcription factors, and immigration and activation of neutrophils. ***The effect of damage control resuscitation strategies on the acute inflammatory response following hemorrhagic shock is unknown.***

Summary. The proposed experiments will increase our understanding of the physiology of damage control resuscitation for hemorrhagic shock. We will examine the effect of varying ratios of plasma to pRBCs in damage control resuscitation on mortality and organ dysfunction after hemorrhage. Additional experiments will determine the effect of damage control resuscitation strategies on activation of pro-inflammatory transcription factors and cytokine production as well as neutrophil activation and organ infiltration after resuscitation. Collectively, these studies will identify the most effective ratio of plasma to pRBCs for resuscitation and will determine the underlying mechanisms of its impact on the development of SIRS and MODS in a murine model of severe hemorrhagic shock.

Preliminary Studies

Hypothesis 1. Damage control resuscitation strategies lead to increased survival and decreased organ injury after hemorrhage. Initial experiments have focused on developing a murine model of hemorrhagic shock and resuscitation. To induce hemorrhage, mice are anesthetized with pentobarbital and each femoral artery isolated and cannulated. Mean arterial blood pressure (MAP) and rectal temperature are constantly monitored. Over a period of 10 minutes, aliquots of blood are removed until the MAP reaches 25 mm Hg. Using return of shed blood as needed, MAP is held constant at 25 mmHg for 60 minutes. Mice are then resuscitated to a MAP of 80 mmHg, decannulated, closed, and allowed to recover. Sham operated animals undergo identical procedures without blood withdrawal.

To determine if there are survival differences between animals resuscitated with fresh whole blood or Ringer's lactate, animals were randomly divided into two groups, then resuscitated with Ringer's lactate solution or fresh whole blood from a donor animal. MAP was similar in each group at baseline, and during hemorrhage and resuscitation (**Figure 1**). Animals were monitored for 1 week after hemorrhage and resuscitation

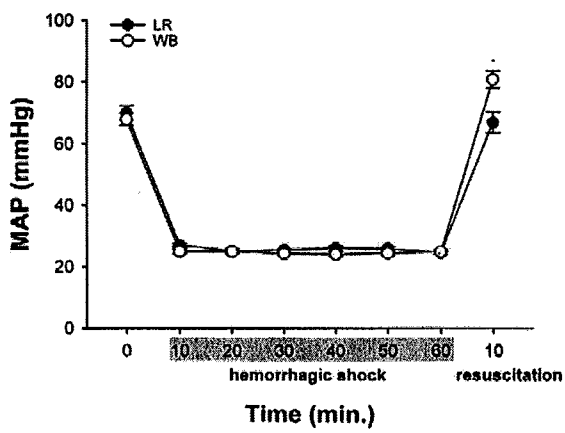

Figure 1. Mean arterial pressure (MAP) during hemorrhage and resuscitation in mice resuscitated with Ringer's lactate (LR) or fresh whole blood (WB). n=10 mice for each condition.

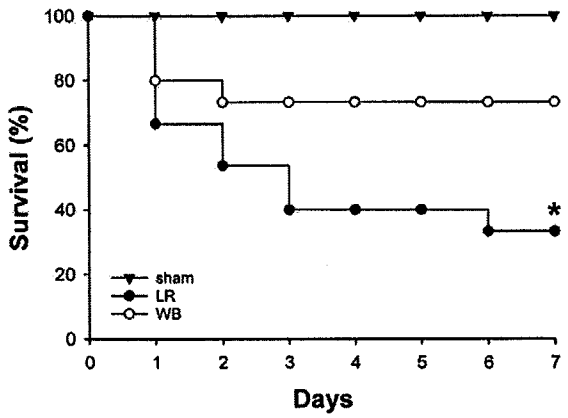

Figure 2. Seven day mortality in mice treated with hemorrhage and resuscitated with Ringer's lactate (LR) or fresh whole blood (WB). n=10 mice for each condition. * $p<0.05$ vs. sham by Kaplan Meier Log rank analysis.

and survival were determined. Mice resuscitated with Ringer's lactate demonstrated increased mortality at 7 days as compared to animals resuscitated with fresh whole blood (**Figure 2**). When infusion volumes were compared, animals resuscitated with Ringer's lactate group required higher volumes of fluid to reach target MAP than did those resuscitated with fresh whole blood, suggesting that these animals demonstrated an increased inflammatory response and capillary leak (**Figure 3**). Together, this data suggests that fresh whole blood is superior to Ringer's lactate for resuscitation from hemorrhagic shock.

Acute lung injury and acute respiratory distress syndrome is a common complication of hemorrhagic shock. We evaluated the effect of resuscitation on lung histology from animals 24 hours after treatment with Ringer's lactate or fresh whole blood. Lungs from mice resuscitated from hemorrhagic shock with fresh whole blood appeared similar to those from animals undergoing sham treatment (**Figure 4**). In contrast, lung sections from animals resuscitated with Ringer's lactate demonstrated alveolar wall thickening, consistent with acute lung injury (**Figure 4**).

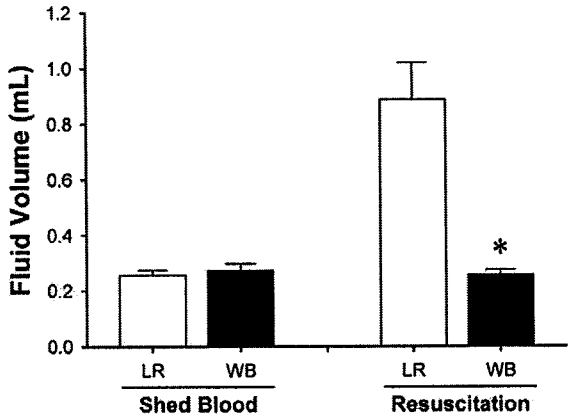

Figure 3. Fluid volume withdrawn during hemorrhage (left bars) and returned as resuscitation (right bars) in mice resuscitated with Ringer's lactate (LR) or fresh whole blood (WB). *$p<0.05$ vs. LR. n=10 mice for each condition.

In order to study the effects of different ratios of plasma to pRBCs, we need to be able to effectively isolate plasma and RBCs from donor animals. We have developed methods that allow us to do this effectively and reproducibly. In preliminary experiments, mice underwent hemorrhage and resuscitation with Ringer's lactate, fresh whole blood, or plasma and pRBCs in a 1:1 ratio. There were no differences in short term survival between animals resuscitated with fresh whole blood or plasma and pRBCs (data not shown). These data demonstrate our ability to utilize blood component therapy for resuscitation. Furthermore, it indicates that mice resuscitated with a 1:1 ratio of plasma to pRBCs have a similar survival response to mice given whole blood.

The effect of damage control resuscitation strategies after hemorrhage on function and architecture of intestine, liver, lung, and kidney is unknown. More importantly, the optimal ratio of plasma to red blood cells utilized for resuscitation is also unknown. Results of these experiments are important as they will guide the ratios of plasma to red blood cells utilized in subsequent studies.

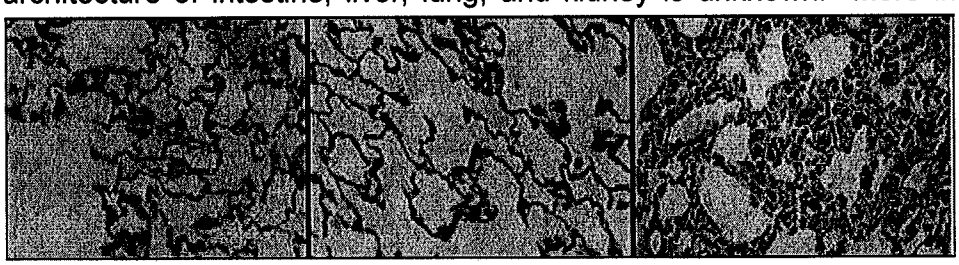

Figure 4. Lung injury 24 hours after hemorrhage and resuscitation.

Hypothesis 2. Damage control resuscitation strategies are associated with diminished pro-inflammatory transcription factor activation and cytokine production after hemorrhage. The induction of a hyperinflammatory state, one of the hallmarks of SIRS, is characterized by activation of pro-inflammatory transcription factors and production of cytokines and chemokines. In an initial set of experiments, we evaluated the effects of resuscitation with Ringer's lactate or whole blood on serum levels of multiple cytokines 1 hour after resuscitation (**Figure 5**). Interestingly, we found no differences amongst the groups for TNF-α or IL-6 at this time, but there were elevations of MCP-1 and MIP-1α in mice resuscitated with Ringer's lactate (n=3). Levels of MCP-1 and MIP-1α in serum of mice resuscitation with whole blood were similar to sham controls. These data suggest that damage control resuscitation reduces the systemic generation of MCP-1 and MIP-1α, two chemokines known to exacerbate SIRS after hemorrhage (13, 14).

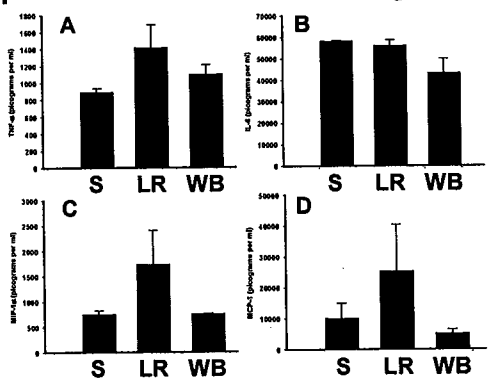

Figure 5. Serum TNF-a (A), IL-6 (B), MIP-1a (C), or MCP-1 (D) 1 hour after sham treatment (S) or hemorrhage and resuscitation with Ringer's lactate (LR) or fresh whole blood (WB).

In a very preliminary experiment, we analyzed the effect of hemorrhage on cytokine production in jejunum after resuscitation with fresh whole blood or Ringer's lactate solution. Forty eight hours after hemorrhage and resuscitation, jejunal IL-6 levels appeared increased in mice resuscitated with fresh whole blood as compared to sham treated animals (**Figure 6**). A further increase in IL-6 levels was seen in mice resuscitated with Ringer's lactate solution. These data represent only an n=1, but if this trend holds true with additional experiments, would suggest that resuscitation with Ringer's lactate leads to an increased intestinal inflammatory response as compared to fresh whole blood.

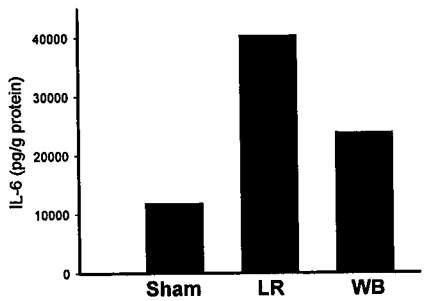

Figure 6. Jejunal IL-6 levels 48 hours after hemorrhage then resuscitation with Ringer's lactate (LR) or fresh whole blood (WB).

We have previously examined NF-κB and AP-1 activation in several different model systems, including endotoxemia and thermal injury (please see biosketch for publications). NF-κB activation is typically rapid, transient, and self limited. For example, in intestinal mucosa, NF-κB activation and IκB-α degradation occur within 60 minutes of treatment with endotoxemia (45; **Figure 7**). In another series of experiments, NF-κB activation and IκB-α degradation in liver were blunted with prior induction of the heat shock response (46; **Figure 8**). Our proposed studies will determine the effect of different resuscitation strategies on organ-specific activation of NF-κB and AP-1. We have substantial expertise in these types of experiments. Similarly, we have experience in measuring mRNA expression in various tissues (46, 47). The effect of damage control resuscitation strategies on mRNA levels of pro-inflammatory mediators in liver, kidney, intestine, and lung is currently unknown. Together, these proposed experiments will identify organ-specific effects of different resuscitation strategies on the inflammatory response to hemorrhage in each organ as well as systemic cytokine levels.

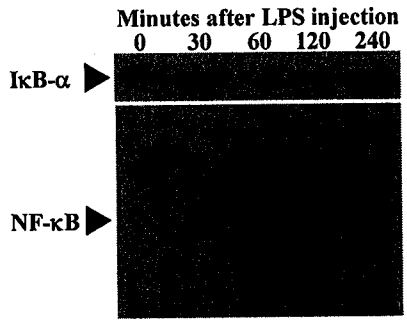

Figure 7. IκB-α degradation and NF-κB activation in jejunum after LPS injection.

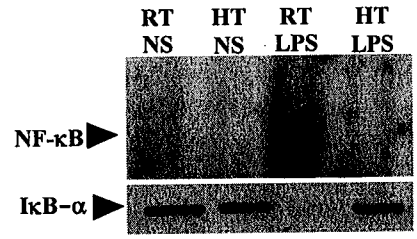

Figure 8. NF-κB DNA binding activity and cytoplasmic IκB-α levels in liver from mice treated with room temperature (RT) or hyperthermia (HT), then saline (NS) or 12.5 mg/kg endotoxin subcutaneously (LPS).

Hypothesis 3. Damage control resuscitation strategies alter neutrophil function after hemorrhage. During the systemic inflammatory response, neutrophils are capable of directly inflicting damage on vital organs and causing organ dysfunction. In preliminary experiments, we analyzed samples from jejunum, liver, and kidney for myeloperoxidase (MPO) activity in order to quantitate neutrophil infiltration. Twenty four hours after hemorrhage and resuscitation, MPO activity was elevated in jejunal samples from mice resuscitated with Ringer's lactate as compared to samples from animals resuscitated with fresh whole blood or sham treated (**Figure 9**). This data suggests that at this time point there is increased neutrophil infiltration into jejunum, but not liver or kidney. This is greatest in mice resuscitated with Ringer's lactate solution. Of note, we have not examined additional time points or MPO levels in lung. Examination of earlier time points will be important as neutrophil infiltration may occur as early as 1 hour after injury and appears to vary in specific organs (48).

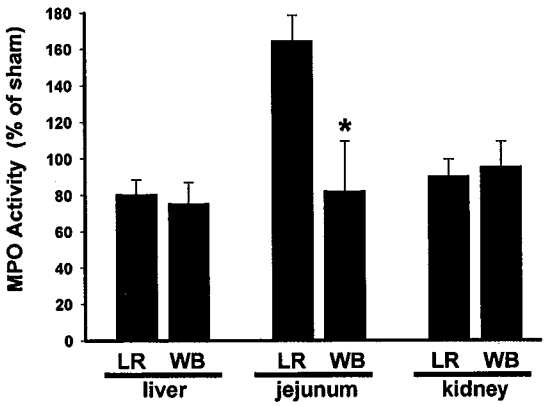

Figure 9. Myeloperoxidase activity in liver, jejunum, or kidney in mice treated with hemorrhage followed by resuscitation with Ringer's lactate (LR) or fresh whole blood (WB). * $p<0.05$ vs. LR.

[redacted] has also established methods to assess the activation of neutrophils from circulating blood as well as from inflamed tissues (49, 50). In this assay, oxidative burst activity is measured in neutrophils isolated from blood and tissues (**Figure 10**). These assays will allow determination of neutrophil activation in peripheral blood and tissues after hemorrhage and resuscitation with Ringer's lactate, fresh whole blood, or damage control resuscitation strategies. The effect of damage control resuscitation strategies on systemic neutrophil activation and function as well as tissue neutrophil infiltration at other time points is unknown. Data from these experiments will be important given the role that neutrophils play in direct organ damage during SIRS and MODS. [redacted], in collaboration with [redacted], has also evaluated the mechanisms of activation of neutrophils during injury. These studies have involved the analysis of the formation of focal adhesion structures (51) and their association with lipid raft domains in neutrophils (52). These assays will be employed in this study and represent new techniques that I am looking forward to mastering and adding to my repertoire.

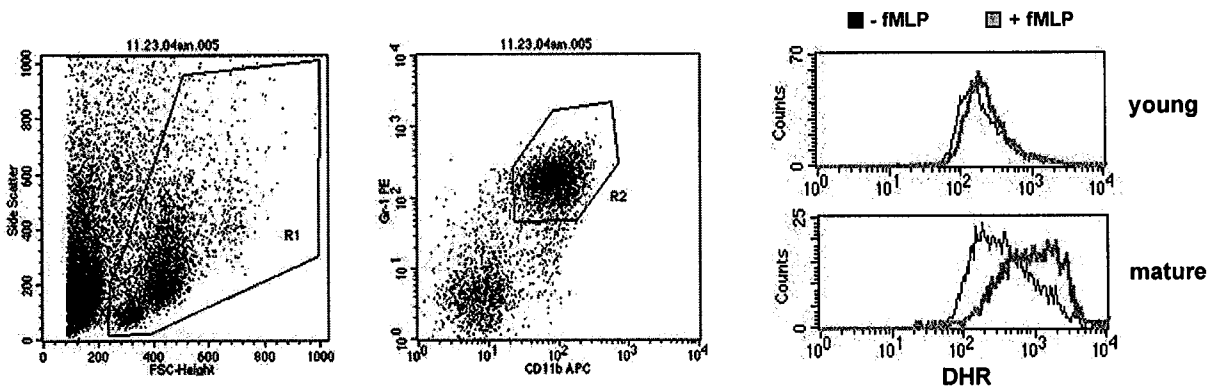

Figure 10. Isolation and analysis of oxidative burst in liver recruited neutrophils. Leukocytes isolated from livers after ischemia/reperfusion were examined by flow cytometry for light scatter and the leukocyte population was gated (left panel; R1). From this population, neutrophils were identified and gated (middle panel; R2) by dual staining with antibodies to Gr-1 and CD11b. Baseline and fMLP-stimulated neutrophil oxidative burst was determined by dihydrorhodamine (DHR) fluorescence using flow cytometry (right panel).

Research Design and Methods

Hypothesis 1. Damage control resuscitation strategies lead to increased survival and decreased organ injury after hemorrhage.

Rationale. Retrospective clinical studies suggest that increased ratios of FFP to packed red blood cells are associated with increased survival after severe hemorrhage (3). While one known benefit of damage control resuscitation is decreased coagulopathy and bleeding, the effect of damage control resuscitation on organ function and host survival is unknown. In addition, the optimal ratio of plasma to pRBCs is unknown. The experiments in this specific aim are designed to examine the effect of resuscitation with Ringer's lactate, fresh whole blood, or varied ratios of plasma to pRBCs on survival and organ function.

Experimental design. We will utilize a murine model of hemorrhagic shock as outlined in the Preliminary Studies section. Our preliminary data suggest that mice subjected to hemorrhagic shock and resuscitated with fresh whole blood have a seven day survival rate of 75% while those resuscitated with Ringer's lactate have a 7 day survival rate of 35%. This provides an ideal setting to determine whether different variations of damage control resuscitation are beneficial or harmful as compared to fresh whole blood or Ringer's lactate.

In order to investigate whether damage control resuscitation strategies alter mortality as compared to Ringer's lactate or fresh whole blood after hemorrhage and resuscitation, mice will be divided into groups and undergo hemorrhage. They will then be resuscitated with different strategies as follows: Ringer's lactate only, fresh whole blood only (no Ringer's lactate), or plasma to RBC ratios of 1:5, 1:3, 1:1, 3:1, or 5:1. Mice will be followed and survival determined out to seven days following injury as mortality during this time period is largely dependent on systemic inflammation and multiple organ failure. These experiments will determine if damage control resuscitation strategies are superior to resuscitation with Ringer's lactate after hemorrhage in regard to survival as well as the optimal ratios of plasma to blood for damage control resuscitation.

Our method to prepare blood and components for therapy is based on previously published protocols (53). In order to obtain blood for resuscitation, donor animals are anesthetized with pentobarbital and blood is withdrawn by sub-xyphoid puncture with a syringe coated with citrate phosphate double dextrose (CP2D) as an anticoagulant. To prepare plasma and pRBCs, CP2D is added to whole blood in a 1:7 ratio and the solution is centrifuged to separate plasma from red blood cells. Once separated, plasma is transfused immediately. The remaining red blood cells are treated with red blood cell nutrient solution (AS-3), analyzed for hematocrit, and utilized as pRBCs. This protocol is similar to that used in human blood banking, including our regional blood bank.

In order to determine the effect of resuscitation with Ringer's lactate, fresh whole blood, or damage control resuscitation strategies on organ function, mice will undergo hemorrhage and resuscitation followed by sacrifice at baseline (shock with no resuscitation), and 1, 2, 6, 24, 48, and 96 hours after resuscitation. Blood is obtained by cardiac puncture and blood gas analysis performed for pH, base deficit, and lactate by autoanalyzer (Radiometer model ABL700, Westlake, Ohio). Base deficit, pH, and lactate are commonly used clinical guides to resuscitation adequacy (reviewed in 54) and will provide important information about the effects of resuscitation strategy on global perfusion and physiology. In order to determine the effect of resuscitation strategies on specific organ function, blood will be analyzed at the same intervals for pO_2 and pCO_2 as indicators of pulmonary function, blood urea nitrogen and creatinine as indicators of renal function, and alanine aminotransferase, aspartate aminotransferase, bilirubin, and lactate dehydrogenase as indicators of hepatic function. Lung, liver, intestine, and kidney will be harvested and analyzed by histology.

Expected results, potential pitfalls, and alternative approaches. Experiments outlined in this aim will begin to delineate the effect of damage control resuscitation strategies on mortality and organ function after hemorrhage. These initial experiments are relatively straightforward in nature but

are necessary given the paucity of data concerning damage control resuscitation strategies as compared to fresh whole blood or Ringer's lactate on survival and organ function following hemorrhagic shock. Based on clinical data, one known beneficial effect of damage control resuscitation is decreased coagulopathy (55). Coagulopathy is not relevant to the hemorrhage model we have chosen and therefore this model allows us to focus on the effects of different resuscitation strategies on the inflammatory response. This is a reductionist approach, but provides a highly controlled, reproducible model system. We feel this is a strength of the proposal. The effect of damage control resuscitation on the inflammatory response to hemorrhage and onset of SIRS is unknown and is the goal of these experiments. We anticipate that fresh whole blood will be superior to Ringer's lactate for resuscitation after hemorrhage in terms of mortality and organ function. While initial results indicate that damage control resuscitation with a 1:1 ratio of plasma to blood is similar to fresh whole blood, the effect of other ratios is unknown. While our model represents a severe hemorrhagic insult, we can modulate the degree of shock to evaluate the efficacy of these strategies on different degrees of shock as they may apply to combat as well as civilian situations. Such studies could represent a logical extension of the proposed studies.

The cause of death in our model is related to SIRS, organ dysfunction and MODS, not coagulopathy. Therefore, differences in mortality between resuscitation strategies will likely be reflective of direct impact on these endpoints. Blood chemistries and organ histology will allow us to determine specific organ dysfunction under these circumstances. Results of these experiments will determine the optimal ratio of plasma to red blood cells for use in damage control resuscitation. We will use this ratio in subsequent experiments.

Hypothesis 2. Damage control resuscitation strategies are associated with diminished pro-inflammatory transcription factor activation and mediator expression after hemorrhage.

Rationale. Previous reports indicate that the pathogenesis of SIRS and MODS after hemorrhage or injury is secondary to dysfunctional inflammation. The effect of damage control resuscitation strategies on the onset of this inflammatory response to hemorrhage is unknown. This series of experiments will determine the effect of hemorrhage followed by resuscitation with Ringer's lactate, fresh whole blood, or the optimum ratio of plasma to RBC (determined in Aim 1) on activation of pro-inflammatory transcription factors and production of pro-inflammatory cytokines.

Experimental design. Mice will undergo hemorrhage and resuscitation with Ringer's lactate, fresh whole blood, or damage control resuscitation followed by sacrifice at baseline, 1, 2, 6, 24, 48, and 96 hours after resuscitation. Blood is obtained by sub-xyphoid puncture. Organs (liver, lung, intestine, and kidney) are procured and snap-frozen in liquid nitrogen for further analysis.

NF-κB and AP-1 are transcription factors that appear to play an important role in the pathogenesis of hyperinflammation after trauma and regulate, at least in part, the transcription of a number of cytokines, chemokines and vascular cell adhesion molecules. Nuclear and cytoplasmic extracts will be prepared from liver, lung, intestine, and kidney. EMSA is performed with specific probes (Santa Cruz Biotechnology, Santa Cruz, CA) to determine NF-κB and AP-1 DNA binding under these conditions. NF-κB activation may be regulated by degradation of its inhibitory protein IκB-α or phosphorylation of the p65 subunit at serine residue 276. To determine IκB-α degradation, cytoplasmic samples are subjected to Western blot using a specific antibody for IκB-α (Santa Cruz). To determine p65 phosphorylation at serine 276, we will perform Western blotting with a phospho-specific anti-p65 ser276 antibody (Cell Signaling). AP-1 activation may occur through two primary mechanisms- either transcriptional activation of Jun and Fos genes or selective phosphorylation of Jun and Fos by MAPK and JNK. Based on results of EMSA assays, we will perform Western blot on candidate samples using antibodies specific for the Jun and Fos family members (Santa Cruz) as well as RT PCR using specific primers in order to determine the mechanism of AP-1 activation. Jun and

Fos phosphorylation will be assayed by Western blot using phospho-specific antibodies (Calbiochem, La Jolla, CA).

To determine the effect of resuscitation with Ringer's lactate as compared to fresh whole blood or damage control resuscitation strategies on systemic and organ specific inflammation, mice will be sacrificed at the time points described above, and serum, liver, lung, intestine, and kidney obtained. In order to determine if these changes are occurring at the transcriptional level, samples from candidate organs will undergo RNA extraction and mRNA analysis using real-time PCR for expression of mRNA for TNF-α, IL-1β, IL-6, MCP-1, MIP-1α, MIP-2, KC, ICAM-1, VCAM-1, and E-selectin using specific custom primers (synthesized by IDT, Coralville, Iowa). Serum and organ specific protein levels of these cytokines and chemokines will be determined by ELISA (reagents from R and D Systems, Minneapolis, Minnesota). Expression of ICAM-1, VCAM-1 and E-selectin proteins will be determined by immunohistochemical staining of tissue sections. These methods are used routinely in [redacted]

Expected results, potential pitfalls, and alternative approaches. These experiments will determine activation of the transcription factors NF-κB and AP-1 as well as serum and organ specific production of proinflammatory mediators after hemorrhage and resuscitation with Ringer's lactate, fresh whole blood, or damage control resuscitation. Since several organs, particularly lung, liver, intestine, and kidney, appear to be involved in the pathogenesis of SIRS and MODS, it will be important to determine transcription factor activation and mediator production in these organs. Based on preliminary data, we anticipate that transcription factor activation and mediator expression will be greater in mice resuscitated with Ringer's lactate as compared to those resuscitated with fresh whole blood. This will be interpreted as evidence that different resuscitation strategies alter the inflammatory milieu on a local and systemic level.

These studies will provide straight-forward readouts that will yield information about the magnitude of inflammatory pathway activation in specific organs. What our proposed studies do not indicate is how we will further investigate the link between resuscitation strategy and the effects observed at the tissue/organ level. We have not included such experiments in our design because they are dependent upon the organ in which differences are observed. For example, based on our preliminary data we have evidence that the lung and gut are sites at which damage control resuscitation may have significant benefit. If these data are validated with further studies, we would design experiments that would assess the manner in which damage control resuscitation protect these organs. An example for the gut might be studies related to maintenance of intestinal barrier function. This would entail in vivo and in situ models of barrier permeability which we have previously employed (57).

We have focused on NF-κB and AP-1 as they appear to be the primary transcription factors regulating the inflammatory response to hemorrhage. However, additional transcription factors, including p38 mitogen activated protein kinase, c-Jun NH_2-terminal kinase, extracellular signal-related protein kinase, and nuclear factor IL-6 also may contribute. If we do not find evidence of NF-κB or AP-1 under these experimental conditions, we will investigate these pathways as well.

Hypothesis 3. Damage control resuscitation strategies alter neutrophil function after hemorrhage.

Rationale. Neutrophils play a key role in the pathogenesis of SIRS and MODS. Different resuscitation strategies, such as hypertonic saline, are known to alter neutrophil activation (58). In response to tissue injury and systemic inflammation, neutrophils contribute to organ damage and dysfunction. The effect of damage control resuscitation on neutrophil function is unknown.

Experimental design. Mice will undergo hemorrhage and resuscitation with Ringer's lactate, fresh whole blood, or damage control resuscitation followed by sacrifice at baseline, 1, 2, 6, 24, 48, and 96 hours after resuscitation. Organ neutrophil recruitment will be determined by tissue histology

as well as by determining myeloperoxidase (MPO) content, a surrogate marker of neutrophil accumulation. These assays are routinely performed in our laboratory and will provide information regarding whether damage control resuscitation alters neutrophil trafficking after resuscitation.

Peripheral blood and organs with significant neutrophil accumulation (based on MPO assay and histology) will be processed for an assay of neutrophil function. This assay is routinely performed in [redacted] and measures oxidative burst by flow cytometry (49, 50). This assay will determine if damage control resuscitation alters the overall activation state of the circulating and organ-recruited neutrophil under different resuscitation conditions. Furthermore, using fMLP as a neutrophil stimulus, we will be able to determine the oxidative reserve of the neutrophil to determine if, for example, an organ recruited neutrophil is able to respond further to a stimulus or whether it has been maximally stimulated.

Previous studies have shown that crystalloid resuscitation activates neutrophils (48). We anticipate that there will be differences in the activation state of neutrophils from mice resuscitated with Ringer's lactate versus damage control resuscitation strategies. Therefore, we will isolate normal neutrophils as previously described (59) and expose them to serum from mice resuscitated with Ringer's lactate, fresh whole blood or damage control resuscitation. We will measure the respiratory burst of these cells using an assay of adherence-dependent production of H_2O_2 as previously reported (52). We will also evaluate the expression and activation of CD11b on the neutrophil surface. This will be performed by flow cytometry using antibodies that recognize total and activated CD11b. We will then evaluate the degree of localization of CD11b to lipid rafts in the neutrophil membrane. This will be determined by isolation of lipid raft domains and Western blot for CD11b, as well as other focal adhesion proteins. Our results from this approach will be supported by immunofluorescent staining of neutrophils with antibodies to the lipid raft marker, GM1, and antibodies to CD11b. These studies will provide a better understanding how different resuscitation strategies affect activation of neutrophils through formation of focal adhesion structures and localization of CD11b to these structures.

Expected results, potential pitfalls, and alternative approaches. These experiments will determine organ specific neutrophil infiltration and activation after hemorrhage and resuscitation with Ringer's lactate, fresh whole blood, or damage control resuscitation strategies. Since several organs, particularly lung, liver, kidney, and intestine are implicated in the pathogenesis of SIRS and MODS, it will be important to determine neutrophil infiltration and activation in these organs. Based on our preliminary data, we anticipate that neutrophil tissue infiltration and activation will be greater in mice resuscitated with Ringer's lactate than those treated with fresh whole blood. The effect of damage control resuscitation on neutrophil tissue infiltration and activation is unknown. If our preliminary experiments are confirmed, it will be interpreted that different resuscitation strategies alter neutrophil tissue infiltration and activation after hemorrhage and may, in turn, exacerbate the onset of SIRS and MODS. These studies will provide information about circulating neutrophil activation as well as organ specific neutrophil infiltration and activation.

Our studies directed at the mechanism by which different resuscitation strategies affect neutrophil activation are not without limitations. First, obtaining "normal" neutrophils from mice will involve isolation from bone marrow. Thus, the studies may be affected by the immature status of some of these cells. Second, the in vitro stimulation of these cells with serum from resuscitated mice is somewhat reductionist approach. However, it will allow us to directly evaluate the different strategies on specific aspects of neutrophil activation. We are hopeful that combined with our results from Hypothesis 2, these studies will provide important new insights into the effects of damage control resuscitation on the neutrophil.