Award Number: W81XWH-11-2-0018

TITLE:"Prevention of Trauma/Hemorrhagic Shock-Induced Mortality, Apoptosis, Inflammation and Mitochondrial Dysfunction"

PRINCIPAL INVESTIGATOR: David John Tweardy, M.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: February 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| February 2015 | Final | 05 Nov 2010 – 04 Nov 2014 |

**4. TITLE AND SUBTITLE**

"Prevention of Trauma/Hemorrhagic Shock-Induced Mortality, Apoptosis,Inflammation and Mitochondrial Dysfunction"

**5a. CONTRACT NUMBER**
W81XWH-11-2-0018

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6.AUTHOR(S)**
**DAVID JOHN TWEARDY, M.D. email: dtweardy@bcm.edu**
Brett Graham, M.D. Michael Norman, M.D.
Adam Abel; Michael Kolosov; Moses Kasembeli, Ph.D.;Zihong Li
Uddalak Bharadwaj, Ph.D.; T. Kristian Eckols; Prema Robinson, Ph.D.; Donti Taraka; Ricardo Siller; Wilson Lau

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

BAYLOR COLLEGE OF MEDICINE
ONE BAYLOR PLAZA
HOUSTON, TEXAS 77030-3498

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland
21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

**Approved for public release; distribution unlimited**

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
We proposed determining if IL-6 merits consideration as a resuscitation adjuvant with Hextend for soldiers suffering from severe battlefield injuries. Our results in rat models demonstrated that IL-6 alone was not sufficient when Hextend is substituted for shed blood as the major resuscitation fluid; rather, soluble IL-6Rα must be given with IL-6. Alternatively, hyper-IL-6, a chimeric protein consisting of IL-6 linked to the soluble IL-6Rα, can substitute for IL-6. Other major findings of our studies were: 1) Leukocyte apoptosis is not sufficiently sensitive to use as a biomarker to guide patient selection for hyper-IL-6, but kidney injury biomarkers may be useful for this purpose; 2) Two proteostasis modulators, Hsp70 and Hsp40, were identified by transcriptome analysis that may mediate the beneficial effects of IL-6 signaling in the liver and lung; 3) In T/HS patients, increased peripheral blood PMN apoptosis is associated with reduced risk of developing infection; 5) IL-6 signaling initiated immediately upon resuscitation in our standard T/HS rat model reduces mortality from *Psuedomonas aeruginosa* pneumonia through maintenance of Surfactant Protein D levels within the lung, which lends further support to the hypothesis that the beneficial effects of IL-6 signaling extends to protection from infection; 6) Mitochondrial function is improved through IL-6 signaling suggesting another mechanism for the apoptosis-reduction benefit of IL-6 signaling, and 7) Stat3β can substitute for Stat3α to restore mitochondrial function in Stat3-deficient cells indicating, for the first time, that both Stat3 isoforms support mitochondrial function.

**15. SUBJECT TERMS**
Hyper (H)-IL-6 as a resuscitation adjuvant. Kidney injury urine biomarkers in trauma/hemorrhagic shock (T/HS). IL-6 signaling reduces pneumonia mortality, increases Hsp70 and Hsp40, and improves mitochondrial function. Stat3 beta supports mitochondrial function.

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 43 | **19b. TELEPHONE NUMBER** *(include area code)* |

**Table of Contents**


**Page**

**Introduction**……..…….…..…..  **4**

**Body**…….. **4**

**Key Research Accomplishments**…….…….. **9**

**Reportable Outcomes**…… **10**

**Conclusion**…… **11**

**References**…… **11**

**Appendices**…… **13**

**Appendix 1**: Figures to accompany Robinson et al manuscript *(pg 13*

**Appendix 2**: Thacker et al manuscript published in *Science Reports (pg 18*

**Appendix 3**: Morrison et al manuscript published in *J Infection (pg 25 ).*

**Appendix 4**: Thacker et al manuscript published in *J Infection (pg 33).*

**INTRODUCTION:**

Trauma complicated by hemorrhagic shock (T/HS) on the battlefield is distinct from the civilian arena especially with regards to clinical diagnosis and resuscitation protocols. Progress towards development of new first-responder resuscitation adjuvants for polytrauma and blast injuries that will maintain tissue viability requires that an agent that demonstrates efficacy in animal models that mimic T-HS and resuscitation in the civilian setting also work in animal models of T-HS that mimic combat casualties and battlefield management. We developed a rat T-HS model in which we demonstrated: 1) 72% mortality at 48 hr, 2) hypovolemic circulatory collapse, 3) left ventricular contractile dysfunction, 4) apoptosis of cardiomyocytes, alveolar epithelial cells, hepatocytes and leukocytes, 5) organ inflammation, 6) organ-specific alterations in the apoptosis transcriptome and 7) increased susceptibility to bacterial infections. Especially notable was the finding that apoptosis and inflammation required resuscitation. Remarkably, use of IL-6 (10 ug/kg) as a resuscitation adjuvant: 1) reduced mortality 5 fold, 2) prevented hypovolemic circulatory collapse, 3) prevented ventricular contractile dysfunction, 4) prevented apoptosis of cardiomyocytes, alveolar epithelial cells, hepatocytes and leukocytes, 5) reduced organ inflammation, 6) normalized the apoptosis and inflammation transcriptome in the heart, lung and liver and 7) reduced T-HS-mediated increased susceptibility to bacterial infections. Importantly from a mechanistic standpoint, results using a pharmacological inhibitor of Stat3 and mice deficient in Stat3β, a dominant-negative isoform of Stat3, demonstrated that virtually all of the beneficial effects of IL-6 were mediated through Stat3 especially Stat3α, which, in addition to its transcriptional role in the nucleus, recently has been demonstrated to support oxidative phosphorylation within mitochondria. Based on these findings (*2-6*), we hypothesize that IL-6 administration at the start of resuscitation will be beneficial to rats and swine subjected to polytrauma and HS models that more closely mimic battlefield injuries and resuscitation protocols and that IL-6 merits consideration as a resuscitation adjuvant for use by medics at the time of Hextend administration to soldiers suffering from T-HS.

We outlined five highly focused Specific Aims to examine this hypothesis:

**Aims 1, 2, and 3.** To determine the effects of IL-6 administration at the start of resuscitation on survival, vital organ apoptosis, injury, inflammation and mitochondrial dysfunction in two rodent models and one swine model that mimic combat casualties and current battlefield fluid resuscitation strategies (Hextend infusion, 14.3 ml/kg):

1) Rats subjected to laparotomy and controlled HS (AIM 1),
2) Rats subjected to femur fracture and T-HS (AIM 2) and
3) Swine subjected to laparotomy, splenectomy, tissue injury and controlled HS (AIM 3).

**Aim 4.** To determine the effects of IL-6 administration on the transcriptome induced by these T-HS models in rats and swine.

**Aim 5.** To determine if leukocyte apoptosis can serve as a biomarker of vital organ apoptosis and injury in T-HS patients.

These studies will establish whether or not the benefits of IL-6 administration will extend to battlefield resuscitation protocols for controlled HS in the setting of moderate to severe trauma and will establish the foundation for clinical trials of IL-6 in civilian injuries involving trauma and HS that mimic severe combat injuries.

**BODY:**

In our original Statement of Work document, we delineated that Tasks in Aims 1 through 5 would be performed in Years 1 through 3 as summarized in the Gantt chart below:

**TIMELINE (GANTT CHART)**

| | **YEAR 1** | **YEAR 2** | **YEAR 3** |
|---|---|---|---|
| **Specific Aim 1.** Effects of IL-6 in rat Lap/HS model | | | |
| | Tasks 1A, B, C and D | | |
| **Specific Aim 2.** Effects of IL-6 in rat FFx/HS model | | | |
| | | Tasks 2A, B, C and D | |
| **Specific Aim 3.** Effects of IL-6 in swine Lap-S-TI/HS model | | | |
| | | | Tasks 3A, B and C |
| **Specific Aim 4.** Effects of IL-6 on rat and swine organ apoptosis and inflammation transcriptomes | | | |
| | | Task 4A | Task 4B / Task 4C |
| **Specific Aim 5.** Leukocytes as a marker of organ apoptosis in T-HS patients | | | |
| | Tasks 5A, B, C and D | | |

These Tasks and the progress we have made in each are outlined below.

***Specific Aim 1. Determine the effects of IL-6 on survival, organ apoptosis, injury and inflammation and mitochondrial dysfunction in rats subjected to Lap/HS model (timeframe: 18 months)***

***Task 1A. Optimize "shock load" for Lap/HS model.***

***Subtask IA1. Amend animal approval to include swine (timeframe=1 month)***

This was postponed.

***Subtask IA2. Determine survival rate of rats subjected to Lap/HS protocol with shock load=60 min (or appropriate; 10 rats; timeframe 1 month).***

In earlier studies performed as part of preliminary studies for this award, we determined that the "shock" load necessary to achieve the target mortality of 50% in the first of our proposed new rat T/HS protocols was 60 min at 35 mm Hg. This protocol includes laparotomy followed by fixed pressure hemorrhagic shock (Rat Lap/HS model). In repeat experiments, we started with a 15-minute period to achieve target MAP of 35 mm Hg followed by 60 min at target MAP. Since there were no deaths, we proceeded to increase the duration of time at target MAP to 75 min, then 90, 120, and 150 and finally 180 min. It was not until using a "shock" load of 150 – 180 min that 50-70% mortality at 48 hr was observed in a total of 6-10 rats examined. Thus, we identified a shock load that allowed us to assess if use of IL-6 as a resuscitation adjuvant provides a survival benefit, as outlined below.

While optimizing the "shock load" for the Rat Lap/HS model, we completed rat studies for a critical experiment at the request of our industry partner (Novartis). Novartis is convinced that prevention of kidney injury is the most compelling pathway for entry into clinical use of human recombinant IL-6 as a resuscitation adjuvant. They have developed a panel of sensitive urine analyte assays to test for kidney injury, which they wanted to use to establish if kidney injury occurred in our standard rat T/HS model and to determine whether kidney injury could be prevented by use of IL-6 as a resuscitation adjuvant. In this experiment, we subjected 6 rats each to either our sham protocol or our standard T/HS protocol, as described (1-3) modified per this grant proposal. Specifically, rats were subjected to trauma (groin incision and bilateral superficial femoral artery cannulation) followed by hemorrhagic shock. Instead of being resuscitated with heparinized shed blood and lactated Ringer's solution, they were resuscitated with Hextend as described in this proposal combined with IL-6 as a resuscitation adjuvant at four doses (0, 3, 10 or 30 mg/kg). Serum was harvested at 24 and 48 hrs; urine was harvested at 4, 12, 18, 24, 36, 42 and 48 hr; and kidneys were harvested at 48. Samples of serum and urine were sent to Novartis on February 23 to be examined in their kidney injury panel. These studies were intended to answer several important questions: 1) Does kidney injury accompany heart, lung and liver injury in rat T/HS? 2) What is the most sensitive and robust urine test to monitor for kidney injury in this setting? 3) Can IL-6 prevent T/HS-induced kidney injury? 4) If so, what is the optimum dose of IL-6 to use in the rat T/HS studies outlined in our proposal?

We received the results from Novartis on August 2, 2011. Of the 10 urine markers of kidney injury, 7 demonstrated evidence of substantial increase including GSTYb, NGAL, Kim-1, Cystatin, beta2M, albumin, and clusterin, which indicated that the kidney demonstrated clear evidence of injury in this model of T/HS. Evidence of injury was maximal by 4 hours. The most sensitive and robust urine marker of kidney injury was Kim-1, but most of the 7 indices were nearly equally sensitive. Much to our surprise, however, while there was some suggestion of protection by IL-6 against renal injury determined by Kim-1 and albumin levels at an IL-6 dose of 10 ug/kg, these results were not statistically significant. Neither was there evidence of benefit of IL-6 on liver and heart injury. The inability to demonstrate a clear benefit of IL-6 at any of the three doses was at odds with all of our earlier publications (1-5). Two major hypotheses for this disparity are: 1) the ability of Hextend to adsorb either IL-6 or sIL-6R, and 2) the absence within the Hextend of a factor(s) present within the returned shed blood (previously used for resuscitation) that are necessary for the beneficial effect of IL-6. The leading candidate for this "missing factor" was sIL-6R.

To explore the hypothesis that Hextend adsorbs either IL-6 or sIL-6R thereby blunting its effects, we examined whether Hextend interferes with the ability of IL-6 alone or in combination with sIL-6R to induce nuclear translocation of GFP-Stat3 in a high-throughput fluorescence microscopy assay we developed to identify small-molecule Stat3 inhibitors (*1*). The results of this study indicated that Hextend had no effect on the potency of either IL-6 or sIL-6R.

To explore the hypothesis that shed blood but not Hextend contains a factor(s) such as sIL-6R necessary to realize the beneficial effects of IL-6, in the fourth quarter, we examined the collected shed blood for the presence of sIL-6R as a function of time of accumulation in our standard T/HS protocol. Our results demonstrated that shred blood accumulated sIL-6R over time to concentrations (100 ng/ml) equivalent to those

of recombinant hIL-6 expected within the circulation of rats receiving the 3 ug/kg dose, the dose we initially reported to be of benefit (*2*).

This finding strongly suggested that the heparinized shed blood was contributing a factor, most likely sIL-6R, that was facilitating the effect of IL-6 and that this factor was absent in Hextend. We designed an experiment consisting of 7 groups of rats @4 rats/group to test this hypothesis:

- **Group 1:** Sham
- **Group 2:** Shock with Hextend resuscitation
- **Group 3:** Shock with IL-6 @ 10 μg/kg with Hextend resuscitation
- **Group 4:** Shock with IL-6 and sIL-6Rα both @ 10 μg/kg with Hextend resuscitation
- **Group 5:** Shock with IL-6 @ 10 μg/kg, shed blood bolus, and 2X total shed blood volume of Ringer's lactated saline (our established resuscitation protocol)
- **Group 6:** Shock with hyper (H)-IL-6 @ 10 μg/kg with Hextend resuscitation
- **Group 7:** Shock with H-IL-6 @ 1 μg/kg with Hextend resuscitation

Groups 6 and 7 will test the sub-hypothesis that H-IL-6, a chimeric protein consisting of human IL-6 fused to the human soluble IL-6 receptor (sIL-6R) α, will be even more potent that the combination of IL-6 and sIL-6Rα. Support for this hypothesis was provided *in vitro* by studies in which we performed a dose-response curve comparing the potency H-IL-6 vs. IL-6/sIL-6Rα (1:1 ratio; **Figure 1**). H-IL-6 was nearly 7-fold more potent that IL-6 + sIL-6R in causing nuclear translocation of GFP-Stat3 in murine embryonic fibroblasts.

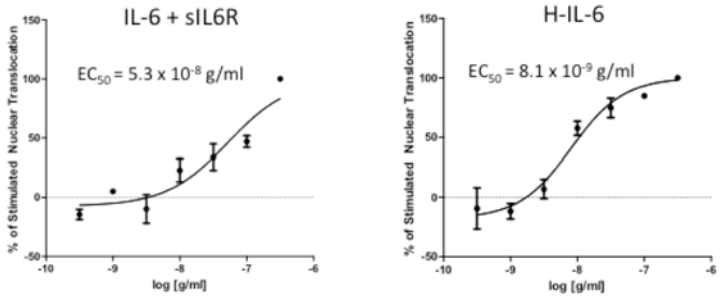



**Figure 1.** Percent of maximum stimulated nuclear translocation of GFP-Stat3 in murine fibroblast cell expressing GFP-Stat3. Assay performed as described (*1*) with increasing concentrations of IL-6 and sIL-6R (left panel) or H-IL-6 (right panel).

Resuscitation-induced apoptosis within the heart (cardiomyocytes), lung (alveolar epithelial cells), and liver (hepatocytes), as determined by TUNEL staining and nucleosome ELISA assays, was prevented in each group that received both IL-6 and sIL-6R (**Appendix 1**), which includes Groups 4, 5, 6, and 7, compared to Group 2. In addition, the liver IL-6 mRNA levels, which are increased 3-fold by T/HS (Group 2 vs. Group 1), return to normal when H-IL-6 (1 μg/kg) is combined with Hextend (Group 7 vs. Group 2); **Figure 2**). Finally, levels of myeloperoxidase (MPO)-positive cell (neutrophil) infiltration into the lung followed as similar pattern (**Appendix 1**). These results confirm the hypothesis that the "missing factor" when Hextend is used as the major resuscitation fluid instead of shed blood is sIL-6R. Furthermore, these findings suggests that hyper (H) IL-6 (1 μg/kg) is fully capable of replacing IL-6 when Hextend is used as a resuscitation adjuvant instead of heparinized whole blood (*7*). We were able to obtain sufficient quantities of hyper-IL-6 from our colleague (Stefen Rose-John) in Germany that allowed us to perform survival experiments as outlined below .

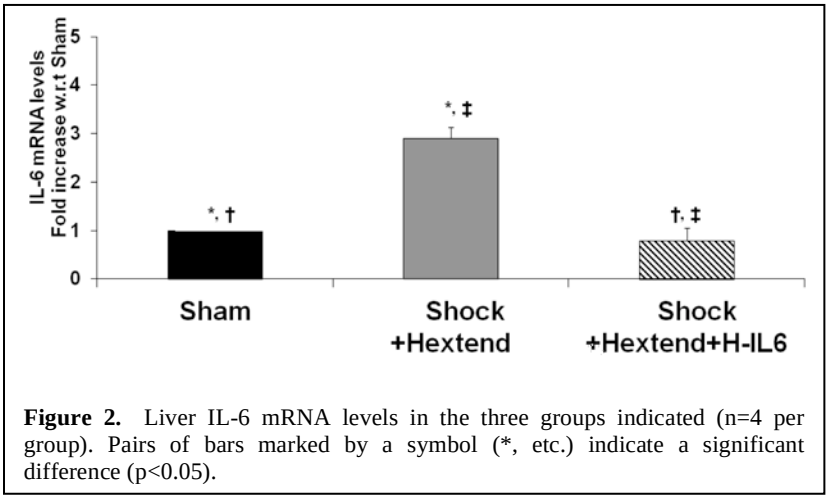



**Figure 2.** Liver IL-6 mRNA levels in the three groups indicated (n=4 per group). Pairs of bars marked by a symbol (*, etc.) indicate a significant difference ($p<0.05$).

***<u>Task 1B</u>. Determine the effect of IL-6 on survival in the rat Lap/HS model.***
***<u>Subtask IB1</u>. Randomly assign rats to the Lap/HS/Hex/P or Lap/HS/Hex/IL-6 groups and observe for 72 hr and record mortality. (20 rats; timeframe=2 months)***

While the Novartis experiment was being analyzed by the company in Year 1, we proceeded with an IL-6 survival study in the Rat Lap/HS model in which the shock load was 180 min at target MAP of 35 mm Hg. Eighteen rats were entered into this protocol and randomized to receive either IL-6 (10 ug/kg in 0.1 ml PBS) or PBS alone as a resuscitation adjuvant. Randomization was performed in such a way that the animal surgeon was blinded to the resuscitation adjuvant each animal received. The PI broke the code on the randomization after 18

rats had been studied. The results demonstrated that while mortality in the IL-6-treated arm (n=10) was 20%, mortality in the placebo arm was only 25%, lower than the 50% expected.

With the findings above strongly suggesting that H-IL-6 can substitute for IL-6 when Hextend is used as the resuscitation fluid, we repeated the experiment substituting H-IL-6 at two doses (1 and 10 μg/kg in 0.1 ml PBS) for IL-6 (**Figure 3**). The results demonstrate that H-IL-6 at 1 μg/kg improved survival from 40% to 75% (p=0.027). The improvement in survival rate in rats resuscitated with H-IL-6 (10 μg/kg) from 40% to 56% did not achieve statistical significance (p>0.05), and were consistent with the findings that the optimum anti-apoptotic effect with H-IL-6 was achieved more consistently within the heart, lung and liver at the 1 μg/kg dose.

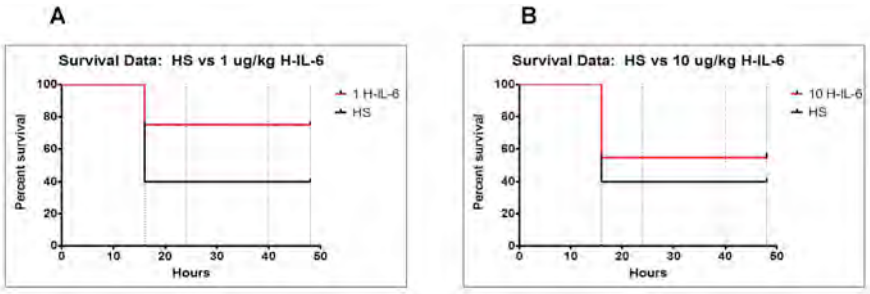



**Figure 3.** Rat surival curves following Lap/HS and resuscitation with Hextend without (HS) or with H-IL-6 (1 or 10 μg/kg).

***Task 1C. Determine the effect of IL-6 on left ventricular contractile function, apoptosis within the heart, lung, liver, kidney, kidney and leukocytes, injury and inflammation within the lung and liver and mitochondrial dysfunction within liver and hearts.***

To lay the foundation for the mitochondrial portion of these studies, we performed a preliminary study to evaluate the potential acute effect of IL-6 administration on mitochondrial function. Three pairs of rats were administered either 10 ug/kg IL-6 (ip) or vehicle (PBS) and one hour later the animals were sacrificed, organs (heart, kidney, liver and lung) harvested, and mitochondria were isolated. The isolated mitochondria were assayed for respiration by polarography, for respiratory chain activities by spectrophotometry, and for relative mitochondrial reactive oxygen species (ROS) levels by measuring native and total reduced aconitase activities. The results of studies on mitochondria isolated from each of the four tissues of IL-6 treated animals were unchanged compared to control-treated animals.

During the past year, we have perfected performing mitochondrial respiration analyses on isolated mitochondria from various rodent tissues as well as on mouse embryonic fibroblasts (MEFs). We have also performed cellular respiration studies on MEFs that completely lack Stat3 (Stat3Δ), express only the Stat3 α isoform (Stat3α), express only the Stat3 β isoform (Stat3β), or express only the Stat3 β isoform that has the last 7 amino acids deleted (Stat3βΔ7). Compared to wild type MEFs, Stat3α and Stat3β cells exhibit normal respiration, while Stat3Δ and Stat3βΔ7 MEFs exhibit significant mitochondrial respiration defects. Mitochondrial electron transport chain enzyme studies demonstrate partial complex I and II deficiencies for Stat3Δ and Stat3βΔ7 MEFs compared to wild type, correlating with the observed respirations defects. Interestingly, when pretreated with 200 ng/mL of IL-6 and soluble IL-6 receptor for 1 hour, the spare respiratory capacity of Stat3Δ MEFs becoming normalized. In addition, the spare respiratory capacity of each of the other cells increased by ~20% raising the possibility that improved cell energetics mediated in part through Stat3 may be responsible for the anti-apoptotic effect of H-IL-6 (*8*).

***Task 1D. Determine the effect of Stat3 inhibition with the GQ-ODN T40214 on IL-6-mediated prevention of T-HS-induced mortality, left ventricular contractile dysfunction, organ apoptosis, injury and inflammation and mitochondrial dysfunction.***

We have developed a better-characterized small-molecule Stat3 inhibitor (*1, 9, 10*) that we will use instead of GQ-ODN T40214.

***Specific Aim 2. To determine the effects of IL-6 administration on survival, vital organ apoptosis, injury and inflammation and mitochondrial dysfunction in rats subjected to FFx/HS model (timeframe: 18 months).***

***Task 2A. Optimize "shock load" for FF/HS model.***

***Subtask 2A2. Determine survival rate of rats subjected to FF/HS protocol.***

Based on the "shock load" determination for the Lap/HS model of 150-180 min, our animal surgeon started with 150 min duration of HS. He did 10 rats @ 150 min, proceeded to 180 min then to 210 min but could not achieve a mortality rate of animals that completed the protocol of >40%. Two issues arose as he

increased the duration of the "shock load": one was that there was unacceptable mortality during the shock phase, the second was that there was variable amounts of bleeding from the femoral fracture site. We opted based on these findings to move from "duration of hypotension" to "percent shed blood return" as a measurement of "shock load". This enabled us to factor in the amount of blood loss from the femur fracture into the "total shed blood" determination and to standardize better across animals for this component of blood "removal". When we established a "shock load" of 35% shed blood return, the mortality achieved 50%, the lower limit of our target.

***<u>Task 2B</u>. Determine effect of IL-6 on survival in the rat FFx/HS model.***

***<u>Subtask IIA1</u>. Randomly assign rats to the FFx/HS/Hex/P or FFx/HS/Hex/IL-6 groups and observe for 72 hr and record mortality.***

As indicated above, hyper (H) IL-6, 1 μg/kg, reduced mortality from our Lap/HS model from 60% to 25% (p=0.027), while H-IL-6 at 10 μg/kg decreased mortality only to 44% (p=0.44). To more clearly establish an optimal dose of H-IL-6 to use as a resuscitation adjuvant with Hextend and to determine if the survival benefit could be seen with use of H-IL-6 as a resuscitation in a HS model involving an even more severe tissue injury than laparotomy, we performed a survival study using a range of H-IL-6 doses that varied roughly in half-log intervals as follows: 0, 0.3, 1, 3 and 10 μg/kg. We also added a non-resuscitated arm to this study. Thus, this study has 6 arms with 10 rats to be entered into each arm. Our animal surgeon was blinded to the dose of H-IL-6 each of the rats is receiving. We completed this study in March 2015 (**Figure 3**). While there was a trend to improved survival with H-IL-6 administration at 1 and 3 μg/kg, the survival in each of these two groups 60%) was not significantly different from the no H-IL-6 group (30%).

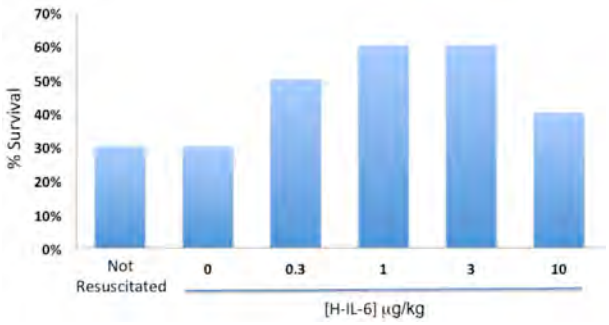



**Figure 3.** Percent survival of rats following the FF/HS/Hex/H-IL-6 protocol.

***Specific Aim 3. To determine the effects of IL-6 administration on survival, vital organ apoptosis, injury and inflammation and mitochondrial dysfunction in swine subjected to laparotomy, splenectomy, tissue injury and controlled HS.***

Based on the results of the study outlined above (**Fig. 3**), we did not proceed with this experiment.

***Specific Aim 4. To determine the effects of IL-6 on the T-HS-induced apoptosis and inflammation transcriptome within the heart, lung, liver, kidney and leukocytes of rats and swine subjected to T-HS and resuscitation protocols (timeframe=12 months; in Year 2).***

Since submission of the proposal in which we presented preliminary data regarding the effect of our standard T/HS protocol on the liver inflammation transcriptome and its beneficial modulation by IL-6, we have published these findings in *PLoS ONE* (*11*). In addition, the PI was invited to give a plenary presentation to the 34th Annual Conference on Shock (Norfolk, VA; 06/12/11; "Contributions of Abnormal Proteostasis to Cellular Dysfunction"). This talk and the data presented therein formed the basis for a peer-reviewed manuscript entitled, "Contribution of the Unfolded Protein Response (UPR) to Hepatocyte and Cardiomyocyte Apoptosis and its Prevention in Trauma/Hemorrhagic Shock", provisionally accepted to *Science Reports* [**Appendix 2**; (*12*)]. This paper reports the first global transcriptome analysis of the UPR in the liver and heart in T/HS; it strongly implicates the non-canonical UPR proteins, heat shock proteins (Hsp) 70 and Hsp40, as modulating hepatocyte apoptosis and mediating protection against apoptosis in response to IL-6. This finding opens new avenues for intervention in the prevention of apoptosis in this setting such as proteostasis modulators. In addition, Stephen Thacker, a talented post-doctoral fellow in the laboratory and first author of this submission, submitted 4 abstracts in 2012 to annual national meetings of the Shock Society, the Infectious Diseases Society of America, the Pediatric Academic Society, and the Pediatric Infectious Diseases Society describing the UPR and it role in alveolar epithelial cell apoptosis in T/HS and its prevention by IL-6.

***<u>Specific Aim 5</u>. To determine if circulating leukocytes can serve as a marker for T-HS-induced apoptosis in T-HS patients (timeframe=16 months).***

***<u>Task 5A</u>. Amend IRB protocol (timeframe=4 months).***

This Task was accomplished.

***<u>Task 5B</u>. Isolate peripheral blood leukocytes from T-HS patients upon entry into the standard vs. hypotensive resuscitation protocol study at the time of randomization (time 0) and at 60-minute intervals until the end of surgery then 1 after the end of surgery and 24 hr after randomization (17 patients; timeframe=10 months).***

This Task was accomplished; see below.

***<u>Task 5C</u>. Perform nucleosome ELISA on leukocyte extracts (timeframe=1 month).***

This Task was accomplished; see below.

***<u>Task 5D</u>. Perform TUNEL of leukocytes (timeframe=1 month).***

Tasks 5B, 5C and 5C were accomplished with the following modifications. Instead of studying only 17 patients, we examined 41 patients. Also, rather than isolating WBC for ELISA and TUNEL at the time of randomization, every hour after randomization, 1 hour after the end of surgery and 24 hours after randomization, we opted to drop the hourly examinations during surgery because of the difficulty in coordinating blood sampling while the patient was undergoing life-saving surgery. Instead, we collected blood for WBC isolation and examinations at the time of randomization, 1 hour after the end of surgery and 24 hour after randomization. Our results demonstrated that there was very little difference in leukocyte apoptosis or any other clinically relevant parameter between these two groups. Consequently, the two group were pooled resulting in the finding [**Appendix 3**; (*13*)] that the circulating leukocytes of T/HS subjects who survived to hospital discharge without developing any infections had significantly higher nucleosome levels 1-hr post-operative compared to those who did develop an infection (49.8 mU/mg protein versus 19.8 mU/mg protein, p=0.02). This difference persisted when analyzing by specific type of infection. TUNEL staining revealed that 72% of apoptotic cells were PMNs. There were no statistically significant correlations between nucleosome levels and survival. Thus, our results indicated that in patients with hemorrhagic shock, increased peripheral blood PMN apoptosis is associated with reduced risk of developing subsequent infection. Previous research has shown that high levels of apoptosis in circulating neutrophils following shock may have a protective effect by preventing neutrophil infiltration and limiting release of harmful oxygen radicals in the tissues. Thus, neutrophil apoptosis may render tissues less susceptible to injury and subsequent infection consistent with strategies aimed at benefiting this patient population by limiting PMN number and aberrant function early in the resuscitation period.

As noted, our results did not demonstrate an association between leukocyte apoptosis and survival. Consequently, leukocyte apoptosis cannot serve as a robust biomarker for predicting patients who may benefit from IL-6 as a resuscitation adjuvant. Given the results above demonstrating the sensitivity of urinary injury biomarkers in our rat T/HS model, we have begun to collect the urine of T/HS patients for measurement of markers of renal injury to establish the best biomarker of renal injury in this patient population to replace leukocyte apoptosis for this purpose. Urine will be collected at the same time points as previously used for blood plus 7 days after randomization.

Thus, all of Aim 5 has been accomplished resulting in a published manuscript (*13*). We made good progress in Aims 1 and 2. We established the requirement for sIL-6R to achieve benefit from IL-6 when Hextend is used for resuscitation, and demonstrated that H-IL-6 could substitute for the combination of IL-6/sIL-6R. The progressive reduction in survival benefit as more tissue injury is added to the HS model suggests that *trans* IL-6 signaling is most beneficial in “pure” hemorrhagic shock injuries.

**KEY RESEARCH ACCOMPLISHMENTS:**

- We demonstrated that kidney injury and apoptosis accompanies heart, lung and liver injury and apoptosis in rat T/HS. Similar to apoptosis in these other organs, kidney apoptosis is prevented when IL-6 is used a resuscitation adjuvant with heparinized shed blood (**Appendix 1**).
- Kidney injury was readily detected within the urine 4 hours after the initiating of resuscitation using 7 of 11 analytes tested by Novartis; 4 of these 7 analytes are available for purchase and use in a Luminex bead-based assay system in the Tweardy lab.
- Results demonstrated that heparinized shed blood accumulated sIL-6R over time to concentrations equivalent to those of recombinant hIL-6 expected within the circulation of rats receiving the 3 ug/kg dose, the dose we initially reported to be of benefit (*2*).
- Studies determined that sIL-6R is an essential factor, in addition to IL-6, for prevention of apoptosis when IL-6 is used as a resuscitation adjuvant in combination with Hextend indicating that IL-6 signaling in *trans* in the predominant pathway through which IL-6 mediates it protective effect.

- Hyper (H)-IL-6 at 1 μg/kg performed better than H-IL-6 at 10 μg/ml in terms of preventing heart, lung, and liver apoptosis and lung inflammation in our standard T/HS protocol. In addition, the lower dose of H-IL-6 also performed better than the higher dose at improving survival from our Lap/HS protocol.
- While there was a trend for improved survival in rats subjected to the FF/HS protocol, these results were not statistically significant. These findings combined with those above suggested that as increasing tissue injury is added to HS, there is reduced benefit of *trans* IL-t signaling in survival protection.
- Leukocyte apoptosis appears not to be sufficiently robust enough biomarker for predicting mortality in T/HS patients.
- However our circulating neutrophil (PMN) apoptosis results indicated that in patients with hemorrhagic shock, increased peripheral blood PMN apoptosis is associated with reduced risk of developing subsequent infection.
- In the first global analysis of the UPR transcriptome ever performed, we identified two non-canonical UPR modulators, Hsp70 and Hsp40, as potential key modulators of liver and lung apoptosis in T/HS that mediate the beneficial effects of IL-6.
- We have made two novel observations regarding the contribution of ligand-mediated Stat3 activation to mitochondrial function. The first is that mitochondrial function is improved through IL-6 *trans* signaling. The second is that Stat3β can substitute for Stat3α to restore mitochondrial function in Stat3-deficient cells.
- In addition to its beneficial effects in preventing organ apoptosis, IL-6 *trans* signaling initiated immediately upon resuscitation in our standard T/HS rat modes also reduces mortality from *Psuedomonas aeruginosa* pneumonia through restoration of Surfactant Protein D levels within the lung (*14*).

**REPORTABLE OUTCOMES:**

**Manuscripts:**

1. Morrison, C.A., Moran, A., Huby M.P., Tweardy, D.J., and Carrick, M.M. Increased Apoptosis of Peripheral Blood Neutrophils is Associated with Reduced Risk of Infection in Trauma Patients with Hemorrhagic Shock. J Infect. 2013 Jan;66(1):87-94. doi: 10.1016/j.jinf.2012.10.001. Epub 2012 Oct 9. PMID: 23063873.
2. Thacker, S. A., Robinson, P., Abel, A., and Tweardy, D. J. (2013) Modulation of the unfolded protein response during hepatocyte and cardiomyocyte apoptosis in trauma/hemorrhagic shock, *Sci Rep 3*, 1187.
3. Thacker, S., Moran, A., Lionakis, M., Mastrangelo, M. A., Halder, T., Huby, M. D., Wu, Y., and Tweardy, D. J. (2014) Restoration of Lung Surfactant Protein D by Il-6 Protects against Secondary Pneumonia Following Hemorrhagic Shock, *J Infect*. 68, 231-241.
4. Robinson, P., Abel, A., Eckols, T. K., and Tweardy, D. J. (2015) IL-6 trans signaling in the immediate post-resuscitation period protects the heart, lung, liver and kidney from apoptosis following trauma/hemorrhagic shock., *PLOS ONE manuscript in preparation*.
5. Taraka, D., Lee, S., Graham, B. H., Dickinson, M., Bharadwaj, U., Abel, A., and Tweardy, D. J. (2015) Contributions of cytokine activation and Stat3 isoforms to mitochondrial function, *PLoS ONE manuscript in preparation*.

**Abstracts:**

1. Thacker SA, Moran A, Huby M and Tweardy DJ. Contribution of Heat Shock Proteins 70 and 40 to Prevention of Alveolar Epithelial Cell Apoptosis in Trauma Complicated by Hemorrhagic Shock. Submitted to the Annual Meeting of the Pediatric Academic Society 2012.
2. Thacker SA, Moran A, Huby M and Tweardy DJ. Contribution of the Unfolded Protein Response to Prevention of Alveolar Epithelial Cell Apoptosis in Trauma Complicated by Hemorrhagic Shock. Submitted to the Annual Meeting of the Pediatric Infectious Diseases Society 2012.
3. Thacker SA, Moran A, Tweardy DJ. Impact of Trauma/Hemorrhagic Shock on the Unfolded Protein Response Transcriptome of the Heart, Lung, and Liver. June 2012 International Federation of Shock Societies Meeting Miami, FL.
4. Thacker SA, Moran A, Huby P, Tweardy DJ. Impaired Host Defense of the Lung Following Trauma with Hemorrhagic Shock: Implicating the Unfolded Protein Response in Alveolar Epithelial Cell Apoptosis. Annual Meeting of the Infectious Diseases Society of American/ID Week, October 2012.

**National Presentations:**

**Oral:**

1. David J. Tweardy. Plenary presentation to the 34$^{th}$ Annual Conference on Shock (Norfolk, VA; 06/12/11; “Contributions of Abnormal Proteostasis to Cellular Dysfunction”).

2. Stephen Thacker, Ana Moran, David J. Tweardy. Implicating the Unfolded Protein Response in Impaired Innate Immunity of the Lung Following Trauma with Hemorrhagic Shock. IDWeek October 2012, San Diego, CA. Oral Presentation.
3. Stephen Thacker, Ana Moran, David J.Tweardy. Contribution of the Unfolded Protein Response to Prevention of Alveolar Epithelial Cell Apoptosis in Trauma Complicated by Hemorrhagic Shock. April 2012 Pediatric Academic Societies (PAS) Boston, MA. Oral Platform Presentation.

**Poster:**

4. Stephen Thacker, Ana Moran, David Tweardy. Impact of Trauma/Hemorrhagic Shock on the Unfolded Protein Response Transcriptome of the Heart, Lung, and Liver. June 2012 International Federation of Shock Societies Meeting Miami, FL. Poster Presentation.
5. Stephen Thacker, Ana Moran, David Tweardy. Contribution of the Unfolded Protein Response to Prevention of Alveolar Epithelial Cell Apoptosis in Trauma Complicated by Hemorrhagic Shock. 2012 St. Jude/PIDS Research Conference, Memphis, TN, February 2012. Poster Presentation.

**CONCLUSIONS:**

We have performed a global transcriptome analysis of the UPR in the liver and heart in T/HS that strongly implicates Hsp70 and Hsp40 as modulating hepatocyte apoptosis and mediating protection against apoptosis in response to IL-6. This finding opens new avenues for intervention in the prevention of apoptosis in this setting such as proteostasis modulators.

We demonstrated in patients with hemorrhagic shock that increased peripheral blood PMN apoptosis is associated with reduced risk of developing subsequent infection. Previous research has shown that high levels of apoptosis in circulating neutrophils following shock may have a protective effect by preventing neutrophil infiltration and limiting release of harmful oxygen radicals in the tissues. Thus, neutrophil apoptosis may render tissues less susceptible to injury and subsequent infection consistent with strategies aimed at benefiting this patient population by limiting PMN number and aberrant function early in the resuscitation period.

Our findings demonstrate that sIL-6R is an essential factor, in addition to IL-6, for prevention of apoptosis when IL-6 is used as a resuscitation adjuvant in combination with Hextend. This requirement can be met by either adding sIL-6R to the IL-6 for use as a resuscitation adjuvant or substituting hyper (H) IL-6 for IL-6 when Hextend is used as the resuscitation fluid. We are collaborate with Stefan Rose-John to commercialize H-IL-6 for this purpose.

**REFERENCES:**


1. Xu, X., Kasembeli, M. M., Jiang, X., Tweardy, B. J., and Tweardy, D. J. (2009) Chemical probes that competitively and selectively inhibit Stat3 activation, *PLoS ONE 4*, e4783.
2. Meng, Z. H., Dyer, K., Billiar, T. R., and Tweardy, D. J. (2000) Distinct effects of systemic infusion of G-CSF vs. IL-6 on lung and liver inflammation and injury in hemorrhagic shock, *Shock 14*, 41-48.
3. Alten, J. A., Moran, A., Tsimelzon, A. I., Mastrangelo, M. A., Hilsenbeck, S. G., Poli, V., and Tweardy, D. J. (2008) Prevention of hypovolemic circulatory collapse by IL-6 activated Stat3, *PLoS ONE 3*, e1605.
4. Moran, A., Akcan Arikan, A., Mastrangelo, M. A., Wu, Y., Yu, B., Poli, V., and Tweardy, D. J. (2008) Prevention of trauma and hemorrhagic shock-mediated liver apoptosis by activation of Stat3alpha, *Int J Clin Exp Med 1*, 213-247.
5. Moran, A., Tsimelzon, A. I., Mastrangelo, M. A., Wu, Y., Yu, B., Hilsenbeck, S. G., Poli, V., and Tweardy, D. J. (2009) Prevention of trauma/hemorrhagic shock-induced lung apoptosis by IL-6-mediated activation of Stat3, *Clin Transl Sci 2*, 41-49.
6. Arikan, A. A., Yu, B., Mastrangelo, M. A., and Tweardy, D. J. (2006) Interleukin-6 treatment reverses apoptosis and blunts susceptibility to intraperitoneal bacterial challenge following hemorrhagic shock, *Crit Care Med 34*, 771-777.
7. Robinson, P., Abel, A., Eckols, T. K., and Tweardy, D. J. (2015) IL-6 trans signaling in the immediate post-resuscitation period protects the heart, lung, liver and kidney from apoptosis following trauma/hemorrhagic shock., *PLOS ONE*, manuscript in preparation.
8. Taraka, D., Lee, S., Graham, B. H., Dickinson, M., Bharadwaj, U., Abel, A., and Tweardy, D. J. (2015) Contributions of cytokine activation and Stat3 isoforms to mitochondrial function, *PLoS ONE*, manuscript in preparation.
9. Redell, M. S., Ruiz, M. J., Alonzo, T. A., Gerbing, R. B., and Tweardy, D. J. (2011) Stat3 signaling in acute myeloid leukemia: ligand-dependent and -independent activation and induction of apoptosis by a novel small-molecule Stat3 inhibitor, *Blood 117*, 5701-5709.

10. Zhang, L., Pan, J., Dong, Y., Tweardy, D. J., Garibotto, G., and Mitch, W. E. (2013) Stat3 Activation Links a C/EBPdelta to Myostatin Pathway to Stimulate Loss of Muscle Mass, *Cell Metabolism 18*, 368-379.
11. Moran, A., Thacker, S. A., Arikan, A. A., Mastrangelo, M. A., Wu, Y., Yu, B., and Tweardy, D. J. (2011) IL-6-mediated activation of Stat3alpha prevents trauma/hemorrhagic shock-induced liver inflammation, *PLOS One 6*, e21449.
12. Thacker, S. A., Robinson, P., Abel, A., and Tweardy, D. J. (2013) Modulation of the unfolded protein response during hepatocyte and cardiomyocyte apoptosis in trauma/hemorrhagic shock, *Sci Rep 3*, 1187.
13. Morrison, C.A, Moran, A., Patel, S., Vidaurre Mdel, P., Carrick, M. M., and Tweardy, D. J. (2013) Increased apoptosis of peripheral blood neutrophils is associated with reduced incidence of infection in trauma patients with hemorrhagic shock, *J Infect* 66, 87-94.
14. Thacker, S., Moran, A., Lionakis, M., Mastrangelo, M. A., Halder, T., Huby, M. D., Wu, Y., and Tweardy, D. J. (2014) Restoration of Lung Surfactant Protein D by Il-6 Protects against Secondary Pneumonia Following Hemorrhagic Shock, *J Infect*. 68, 231-241.

**APPENDICES:**

**Appendix 1**: Figures to accompany Robinson et al manuscript.

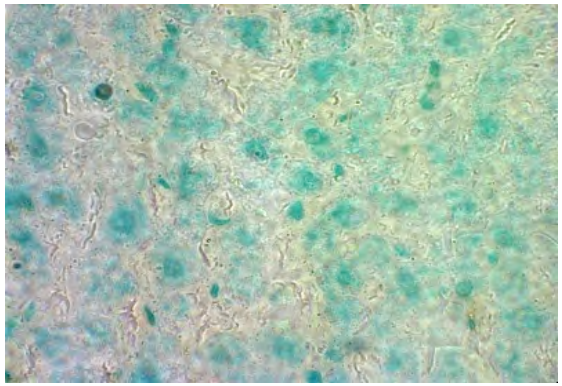
**Appendix 2**: Thacker et al manuscript published in *Science Reports*.

**Appendix 3**: Morrison et al manuscript published in *J Infection.*

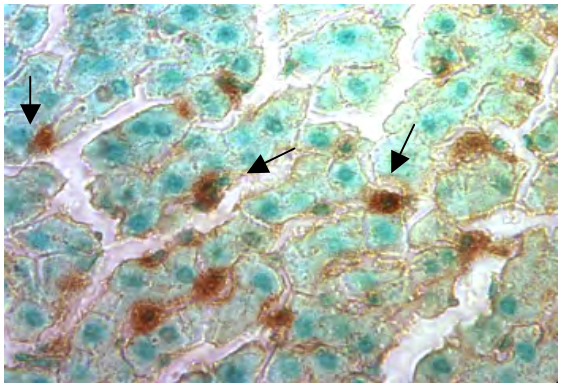
**Appendix 4:** Thacker et al manuscript published in *J Infection.*

**LIVER**

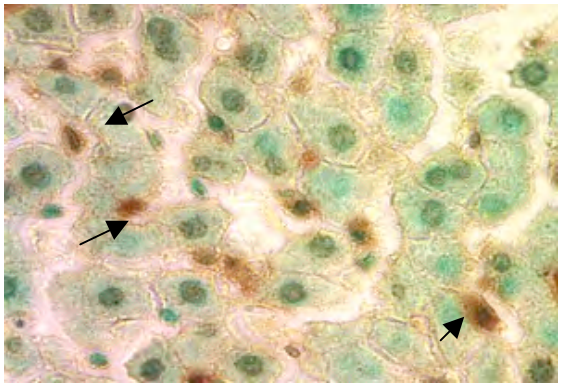
SHAM



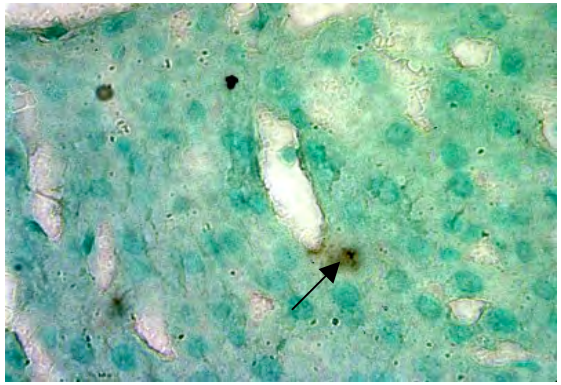
HS+HEXTEND



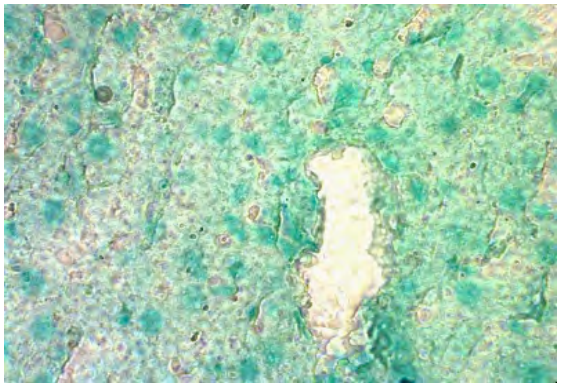
HS+HEXTEND+IL-6



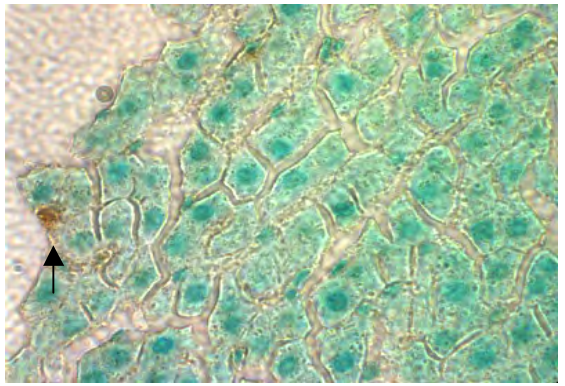
HS+HEXTEND+IL-6+sIL-6R



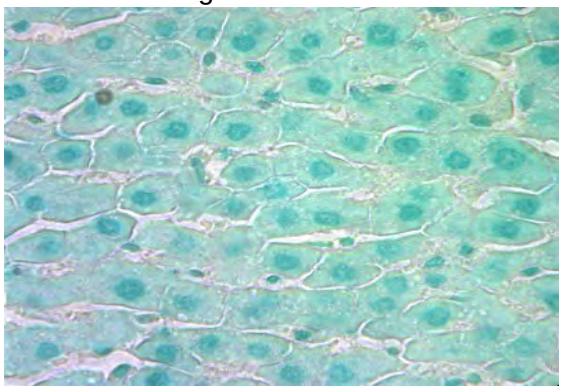
HS+IL-6+Shed blood

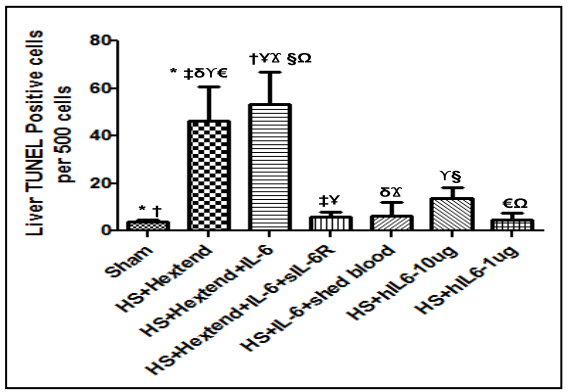


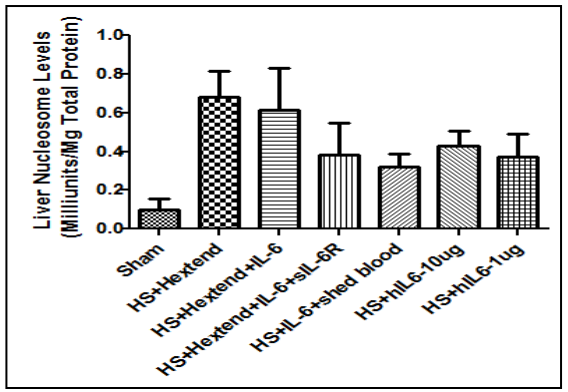
HS+hIL-6-10ug



HS+hIL-6-1ug

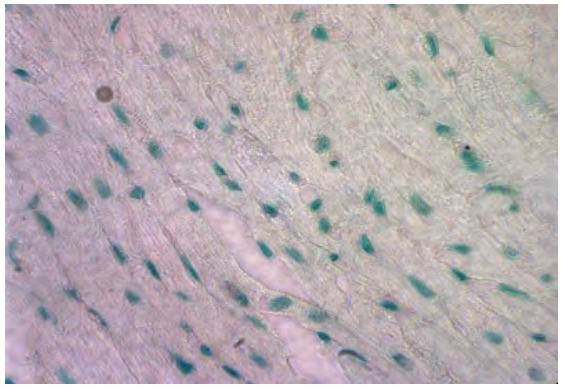




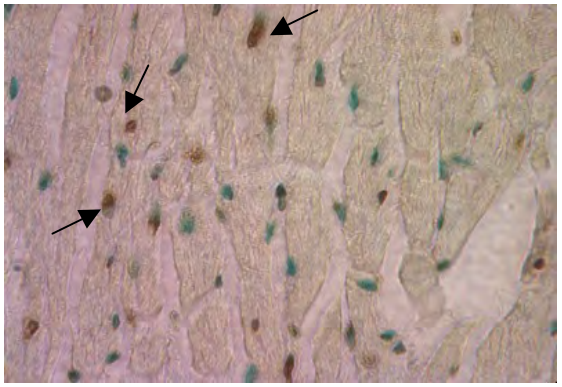



# HEART

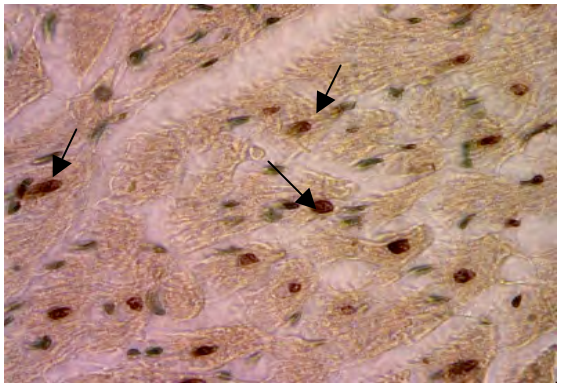
SHAM



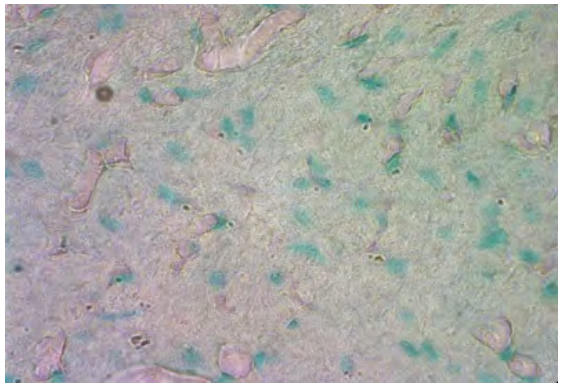
HS+HEXTEND



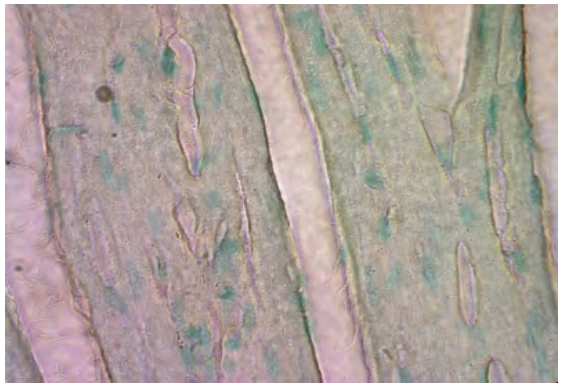
HS+HEXTEND+IL-6



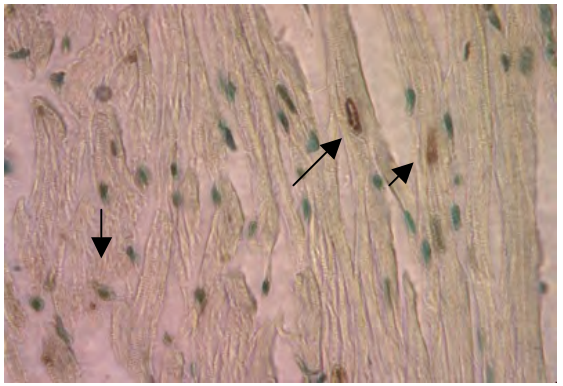
HS+HEXTEND+IL-6+sIL-6R



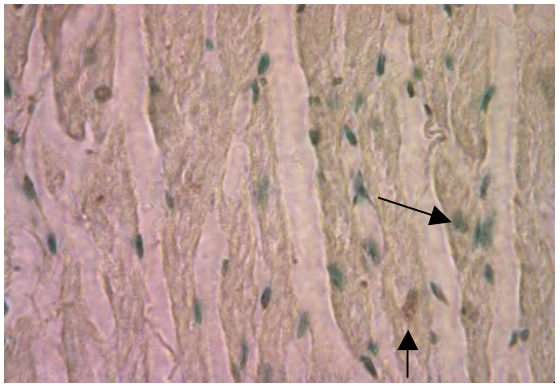
HS+IL-6+Shed blood

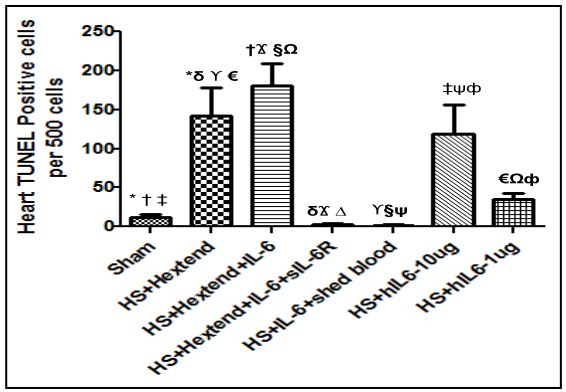


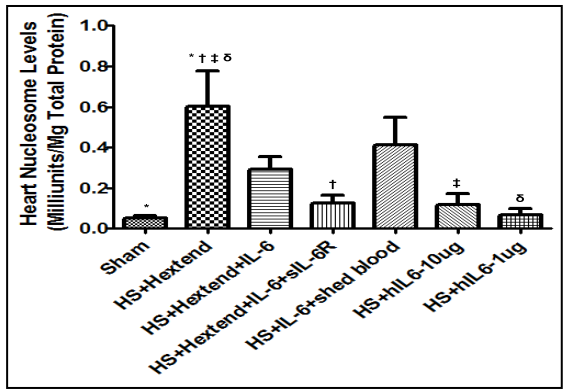
HS+hIL-6-10ug



HS+hIL-6-1ug

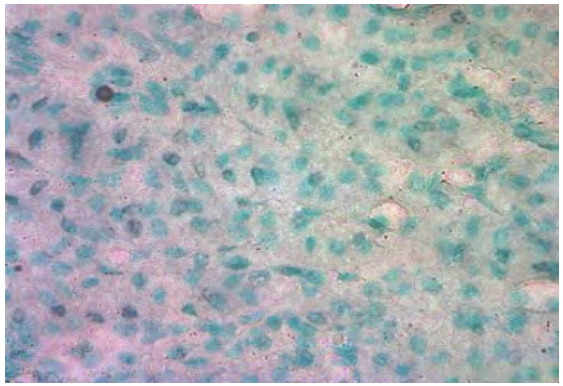




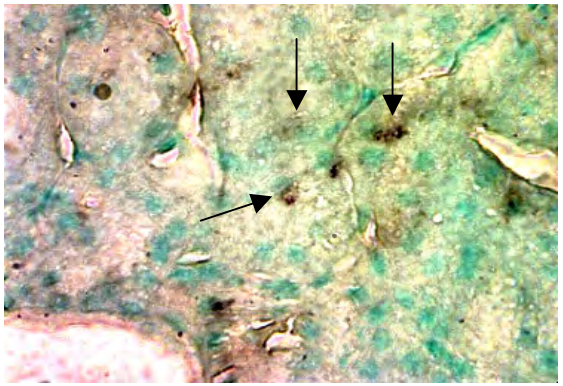



**KIDNEY**

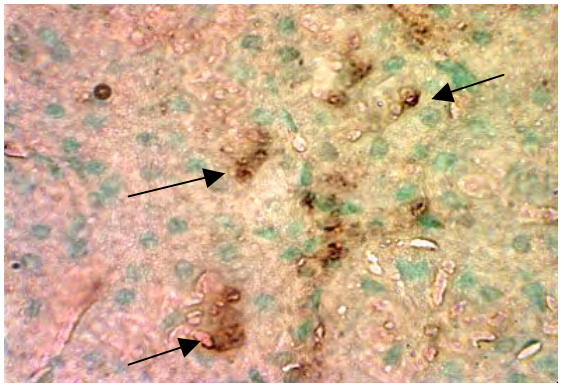
SHAM



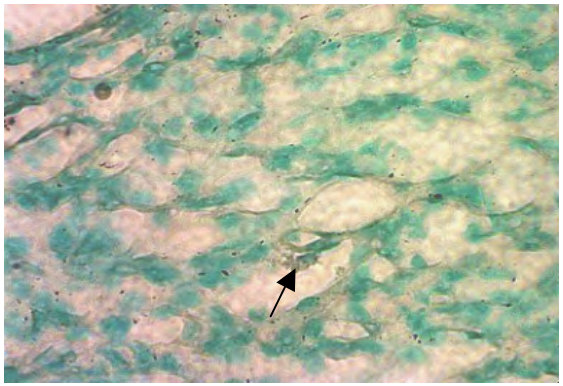
HS+HEXTEND



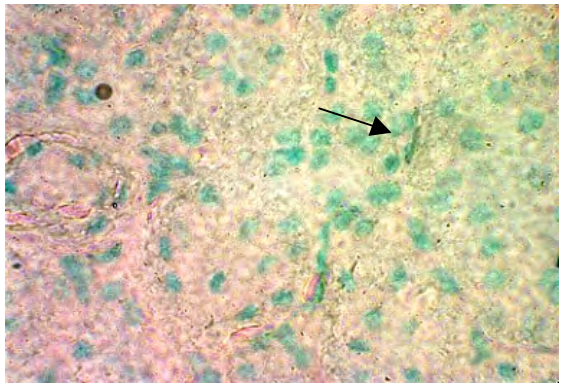
HS+HEXTEND+IL-6



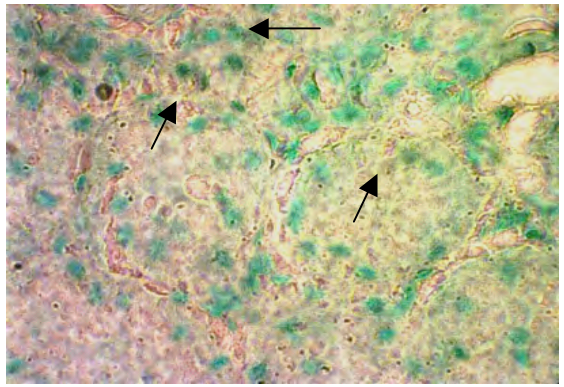
HS+HEXTEND+IL-6+sIL-6R



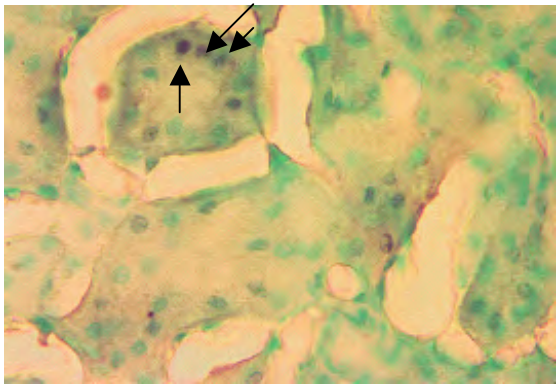
HS+IL-6+Shed blood

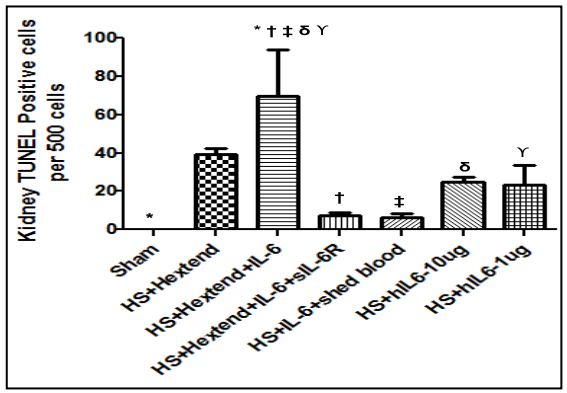


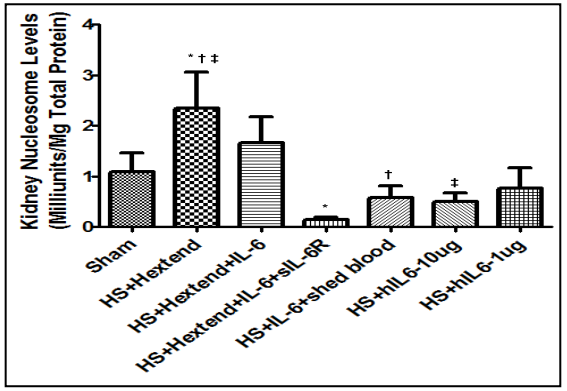
HS+hIL-6-10ug



HS+hIL-6-1ug

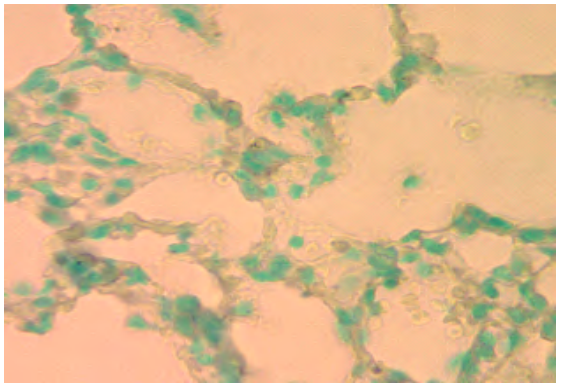




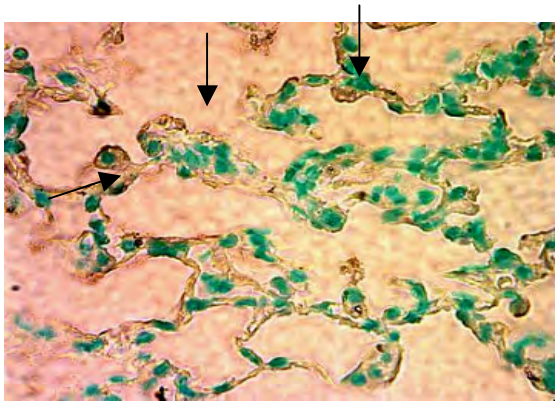



**LUNG**

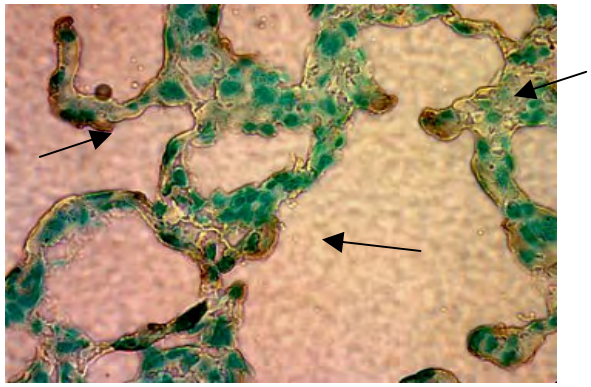
SHAM



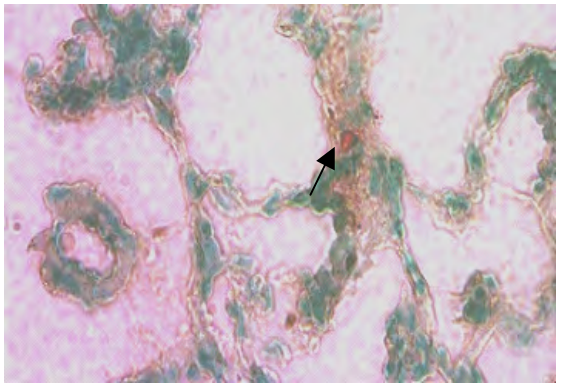
HS+HEXTEND



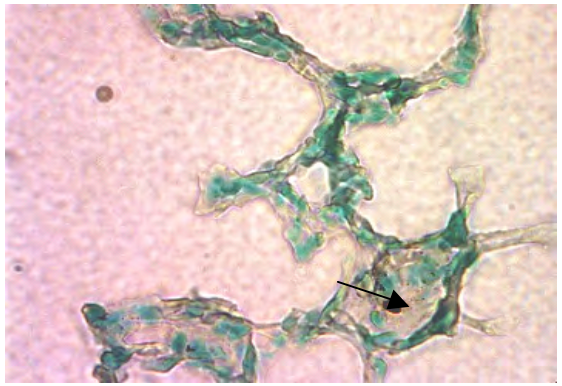
HS+HEXTEND+IL-6



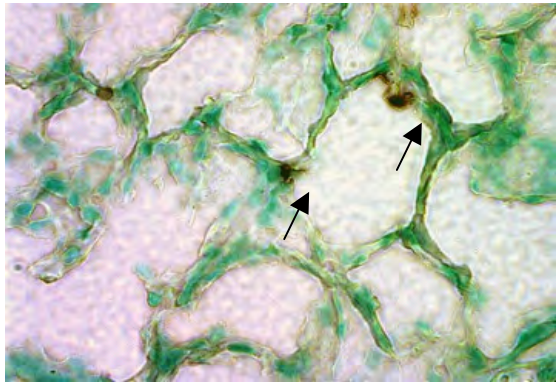
HS+HEXTEND+IL-6+sIL-6R



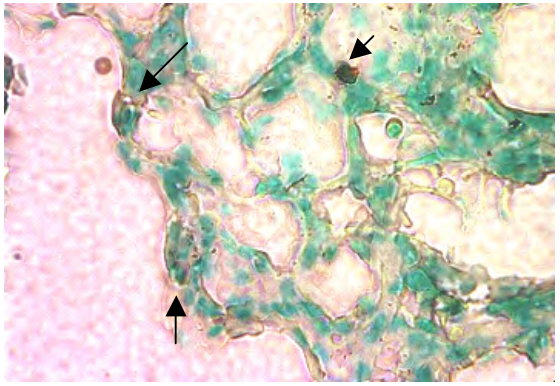
HS+IL-6+Shed blood

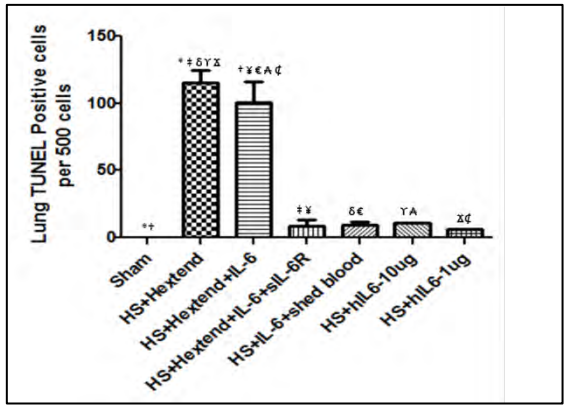


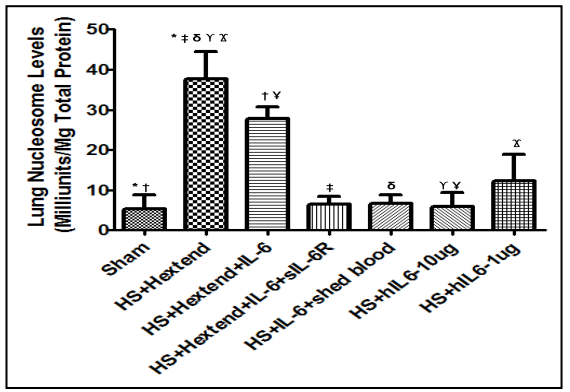
HS+hIL-6-10ug

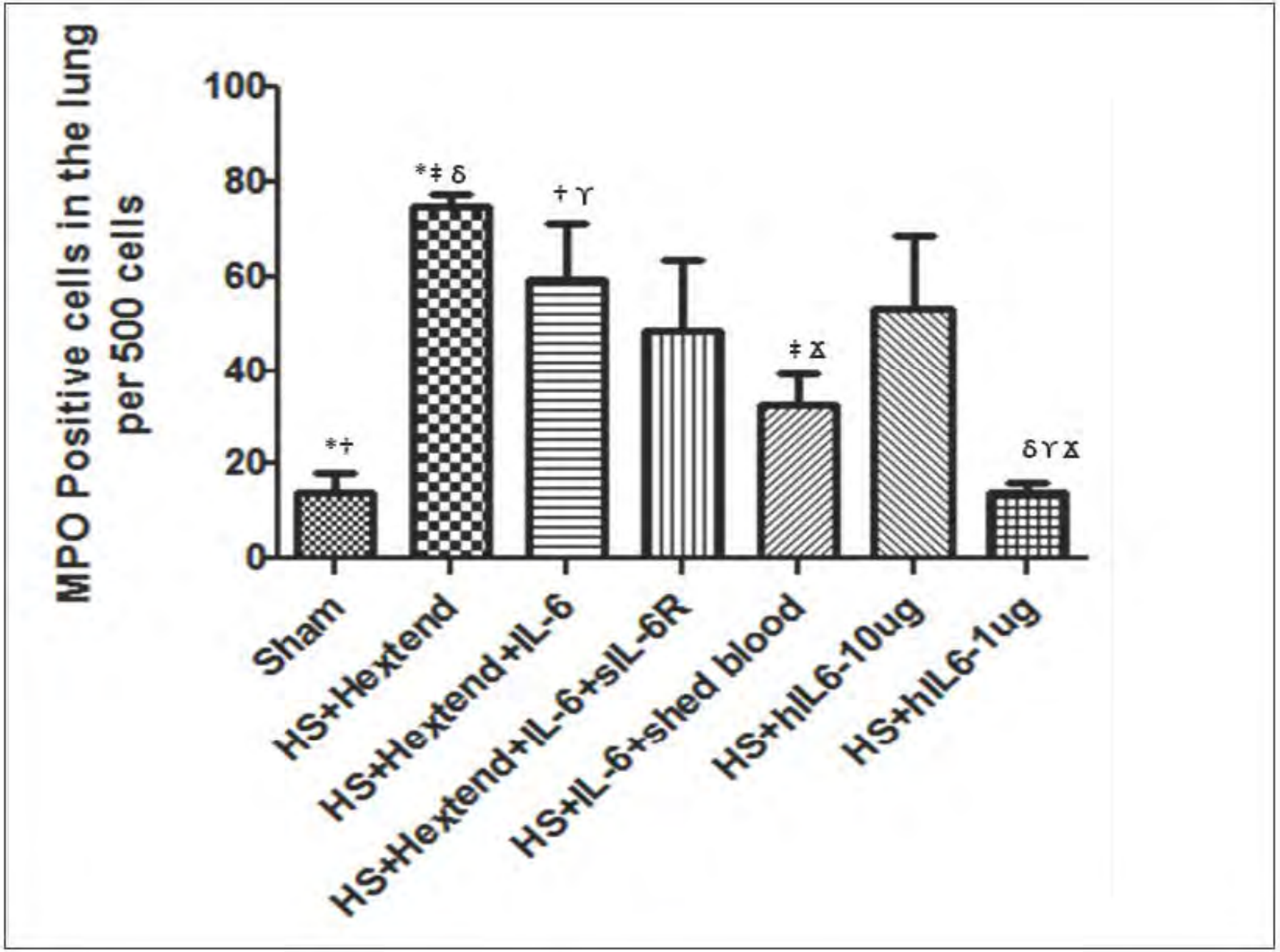


HS+hIL-6-1ug






MPO Positive cells in the lung per 500 cells
100
80
60
40
20
0
*†
*‡δ
†γ
‡ϰ
δγϰ
Sham
HS+Hextend
HS+Hextend+IL-6
HS+Hextend+IL-6+sIL-6R
HS+IL-6+shed blood
HS+hIL6-10ug
HS+hIL6-1ug

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
ENDOPLASMIC RETICULUM
APOPTOSIS
TRAUMA
ANIMAL DISEASE MODELS

# Modulation of the Unfolded Protein Response During Hepatocyte and Cardiomyocyte Apoptosis In Trauma/Hemorrhagic Shock

Stephen A. Thacker[1], Prema Robinson[2], Adam Abel[2] & David J. Tweardy[2]

[1]Section of Infectious Diseases, Department of Pediatrics, [2]Section of Infectious Diseases, Department of Medicine; Baylor College of Medicine, Houston, Texas.

Received
25 October 2012

Accepted
9 January 2013

Published
1 February 2013

Correspondence and requests for materials should be addressed to D.J.T. (dtweardy@bcm.edu)

**Trauma with hemorrhagic shock (T/HS), has been shown to result in liver injury marked by hepatocyte apoptosis and heart failure marked by cardiomyocyte apoptosis, both of which we have shown to be prevented by IL-6 administration at resuscitation, and Stat3 largely mediated this. As specific mediators have not been delineated, we investigated the unfolded protein response (UPR), which, with marked activation, can lead to apoptosis. Prior studies of hepatic and cardiac injury examined limited repertoires of UPR elements, making it difficult to assess the role of the UPR in T/HS. This study describes the first global examination of the UPR transcriptome in the liver and heart following T/HS, demonstrating organ-specific UPR transcriptome changes. The non-canonical UPR chaperone, Hsp70, was most dysregulated following T/HS and may contribute to hepatocyte protection via an IL-6-mediated pathway, identifying a potential new therapeutic strategy to prevent hepatocyte death and organ dysfunction in T/HS.**

Trauma is a leading cause of morbidity and mortality in the United States for those under the age of 45 years, especially when complicated by hemorrhagic shock[1]. When trauma with hemorrhagic shock (T/HS) is accompanied with resuscitation, the end effect is essentially a systemic ischemia and reperfusion injury. Multiple organ failure is an important maladaptive sequelae contributing to late mortality in those who survive beyond 24 hrs following severe T/HS and resuscitation[2].

Work done by our group, and others, in rodent models of T/HS, has shown that parenchymal cells within organs such as the liver, a key metabolic and homeostatic organ, and heart, an organ whose dysfunction often heralds post-traumatic mortality, undergo apoptosis[3–7]. The pathways leading to parenchymal cell apoptosis in these organs in T/HS are not fully understood. The classical mechanisms of apoptosis, such as the extrinsic and intrinsic apoptotic pathways, have been investigated in the liver and heart[3,6]. However, specific delineation of the pathways leading from T/HS to cell death and organ dysfunction is incomplete.

Prolonged or severe endoplasmic reticulum (ER) stress has recently been demonstrated to lead to apoptosis through the unfolded protein response (UPR). The canonical genes involved in ER stress and the UPR were first delineated in yeast including identification of the ER membrane bound sensors of ER stress[8–11]. Homologues for these sensors and their targets have been identified in mammals and their activation can reliably be assessed transcriptionally. While much of the focus of investigation on the UPR has centered around the three main signaling molecules inositol-requiring enzyme 1α (IRE1α), Activating Transcription Factor 4 (ATF4), and protein kinase RNA-like endoplasmic reticulum kinase (PERK), many non-canonical modulators of the UPR have been identified linking the UPR to pathways ranging from innate immunity to apoptosis. Emerging evidence has shown that prolonged ER stress and UPR activation leads to apoptosis that is an important mechanism of disease pathogenesis in a number of genetic disorders, such as lysosomal storage diseases, particularly within the liver[12,13]. Examination of the UPR as a potential cause of parenchymal cell apoptosis in metabolic and other derangements leading to ER stress initially focused on exocrine organs such as the liver[14]. The UPR and its contribution to liver disease has been investigated in liver diseases such as steatosis[15,16], ischemia/reperfusion injury[17,18] and T/HS[19,20]. The impact of the ER stress and the UPR on non-exocrine organs such as the heart, has only recently become a focus[21,22]. Studies of both the liver and heart are limited, however, since they have focused on isolated components of the UPR and did not provide direct evidence that would allow one to conclude that

Table 1[§] | Impact of T/HS without and with IL-6 on markers of apoptosis in the heart and liver

| | Liver | | Heart | |
|---|---|---|---|---|
| Intervention | Nucleosome[a] | TUNEL[b] | Nucleosome[a] | TUNEL[b] |
| Sham | 139 ± 67* | 0.9 ± 0.4** | 0[‡] | 1.3 ± 0.2[‡‡] |
| T/HS | 1874 ± 127*† | 27 ± 3.6**,†† | 63 ± 8[Δ,‡] | 16.2 ± 2[Δ,‡‡] |
| T/HS-IL6 | 264 ± 36†¥ | 1.9 ± 0.5††,¥¥ | 4 ± 1[◇,Δ] | 8.5 ± 0.2[◇◇,ΔΔ] |
| T/HS-IL6-GQ | 1556 ± 241¥ | 12.3 ± 1.1¥¥ | 24 ± 5[◇] | 16.5 ± 1[◇◇] |

[§] *,†,¥,**,††,¥¥,‡,Δ,◇,‡‡,ΔΔ,◇◇ indicate group comparisons with statistical significance of $p < 0.05$, one-way ANOVA.
[a]Nucleosome data presented as mU/mg total protein.
[b]TUNEL data presented as number of TUNEL-positive nuclei per high power field.

apoptosis or organ injury resulted from an insufficient adaptive UPR or that the UPR or components therein were, in fact, maladaptive.

We previously demonstrated that parenchymal cell apoptosis following T/HS in both the liver and heart is prevented by administration of IL-6, which mediates its effect through the actions of Stat3[3,6]. In the current studies, we performed UPR transcriptome analysis of the liver and heart at a global level to identify candidate genes within the canonical and non-canonical UPR that contribute to apoptosis following T/HS. By tracking the direction and magnitude of changes in levels of these candidate genes that occurred following T/HS with IL-6 resuscitation, with or without Stat3 inhibition, we were able to clearly identify those genes most implicated in T/HS-induced apoptosis and its prevention by IL-6-activated Stat3. In particular, we demonstrated that Hsp70 and 40 were upregulated in the liver by T/HS, and that this response was adaptive and insufficient since IL-6 augmented it, thereby preventing apoptosis.

## Results

**T/HS-induced hepatocyte apoptosis is prevented by IL-6 resuscitation; the IL-6 effect is mediated, in part, by Stat3.** To confirm our previous findings that T/HS induces liver apoptosis, we measured histone-associated DNA fragments (nucleosomes) in the livers of rats subjected to our T/HS protocol. Nucleosome levels were 13.5 times higher than sham ($p < 0.001$, ANOVA; Table 1). The nucleosome results were confirmed by TUNEL staining (Table 1), which also demonstrated that hepatocytes represented the overwhelming majority of cells undergoing apoptosis (data not shown).

Nucleosome levels in the IL-6-resuscitated rats were decreased 7.1 times compared to those of the T/HS group ($p < 0.001$) and were similar to sham levels (Table 1). TUNEL staining confirmed these results (Table 1). The number of TUNEL-positive nuclei/hpf in the IL-6 group was decreased 14.2 times compared to the placebo group ($p < 0.001$), to levels statistically similar to those of the sham group (Table 1).

Pretreatment of rats with the G-rich, quartet-forming oligonucleotide Stat3 inhibitor (T40214) was accompanied by a return of nucleosomes to levels similar to those of the placebo treated group and 5.9 fold higher that those of the IL-6 treated group ($p < 0.001$; Table 1). Similarly, the number of TUNEL-positive nuclei/hpf in livers of rats from the T/HS-IL6-GQ group was 6 fold higher than that of the T/HS-IL6-treated group ($p < 0.0001$); Table 1). Nucleosome levels and number of TUNEL-positive nuclei/hpf in livers of rats pre-treated with a NS-ODN before T/HS and IL-6 resuscitation were indistinguishable from those of the IL-6 group (data not shown). Thus, pharmacological inhibition of Stat3 using T40214 in rats subjected to severe HS resuscitated with IL-6 completely blocked IL-6-mediated prevention of liver apoptosis.

**Liver UPR transcriptome is significantly altered in T/HS.** We investigated the impact of T/HS on the ER stress response at the transcriptome level, and then defined the role of this ER stress response on the observed reversible hepatic apoptosis. Unbiased hierarchical clustering of our experimental animals based on intervention group and entity clustering with the UPR transcriptome demonstrated the reproducible nature of the impact of T/HS on the UPR transcriptome (Figure 1). Of the broad 185-gene UPR-associated entity list generated via literature review and Ingenuity Pathway Analysis (IPA®), 113 distinct gene entities were annotated and expressed across our chips after spot duplicates were removed. Using this list of 113 genes, 63 (56%) were significantly altered in one-way ANOVA ($p < 0.05$) among all three-group comparisons, T/HS vs. Sham, T/HS-IL6 vs. T/HS, and T/HS-IL6-GQ vs. T/HS-IL6. When the impact of T/HS was looked at specifically, 31 (27%) of those gene entities were significantly dysregulated in the T/HS group when compared to sham, with 55% (17 of 31) significantly upregulated and 45% of gene transcripts downregulated. When asking the question of potential mediators of the protective effect of IL-6, 17 entities were significantly altered in both group comparisons. Taking known apoptotic function of these genes into context, we demonstrated that all UPR-associated genes with known potential

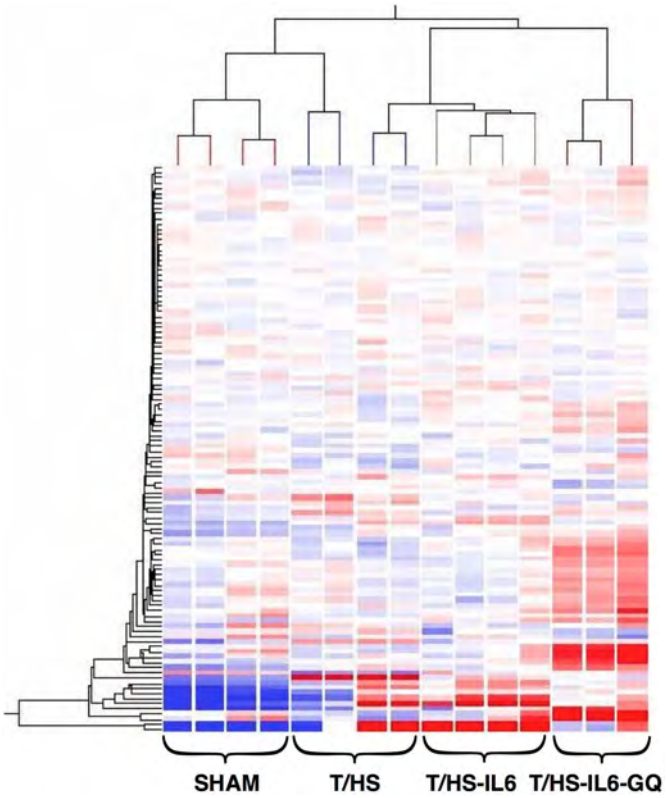



**Figure 1 | Unbiased hierarchical heatmap clustering based on both UPR entity and experimental intervention of animals confined to 113 UPR-associated gene entities on whole liver preparations.** Clustering performed using Hierarchical analysis using Euclidean similarity measure, expression data normalized to chip standards for clustering.

Table 2 | Liver UPR Transcripts Significantly Altered in Both T/HS vs. Sham and T/HS/IL6 vs. T/HS Comparisons

| Gene Symbol | UPR Function | Apoptosis Function | Regulation | | Fold Change | |
|---|---|---|---|---|---|---|
| | | | T/HSvs Sham | T/HS-IL6 vs T/HS | T/HS vs Sham | T/HS-IL6 vs T/HS |
| Hspa1a/Hspa1b (Hsp70) | Chaperone | Anti | up | up | 25.6 | 5.5 |
| Hspa1b (Hsp70-1b) | Chaperone | Anti | up | up | 18.4 | 5.9 |
| Ero1l | Disulfide Bond Formation | Anti | up | down | 9.8 | −3.8 |
| Dnajb1 (Hsp40 Subunit b1) | Chaperone | Anti | up | up | 5.9 | 2.7 |
| Atf4 | Transcription Factor | Anti/Pro | up | down | 3.1 | −2.0 |
| Casp3 (caspase 3) | Apoptosis Signalling | Pro | up | down | 1.8 | −1.7 |
| Eif2s1 (Eif2α) | Protein Transtation | Anti | up | down | 1.7 | −1.6 |
| Sels | Modulation of ATF6 | Uknown | up | down | 1.5 | −1.3 |
| Eif2ak3 (PERK) | UPR Sensory Molecule | Anti/Pro | up | down | 1.4 | −1.3 |
| Psmb3 | Proteasome Degradation | Anti | down | up | −1.2 | 1.3 |
| Calr (calreticulin) | Chaperone | Anti | down | up | −1.2 | 1.2 |
| Uba1 | Ubiquitination | Anti | down | up | −1.3 | 1.2 |
| Psme2 | Proteasome Degradation | Anti | down | up | −1.3 | 1.4 |
| Psme1 | Proteasome Degradation | Anti | down | up | −1.3 | 1.2 |
| Dyt1 | ATPase | Anti | down | up | −1.4 | 1.4 |
| Tmbim6 (Baxinhibitor 1) | Apoptosis Signalling | Anti | down | up | −1.5 | 1.3 |
| Ccnd1 | Cell Cycle Signalling | Anti | down | up | −3.4 | 2.3 |

pro-apoptotic function were upregulated following T/HS and subsequently normalized with IL-6 (Table 2). The most dysregulated genes within this intergroup comparison were the chaperones, Heat Shock Protein 70 (25.6-fold), and Heat Shock Protein 40 (5.9-fold), the UPR transcription factor ATF4 (3.1-fold), and endoplasmic oxidoreductin-1-like protein (Ero1l) (9.8-fold) suggesting a strong impact on the protein folding mechanics both in the cytoplasm and the endoplasmic reticulum. Indeed, when assessed by Real-Time PCR (RTPCR), Hsp70 and Hsp40 demonstrated significantly increased transcript levels in T/HS animals when compared to Sham, with 5.1 fold ($p = 0.004$) and 3.5 fold ($p = 0.001$) increase, respectively (Figure 2). Likewise confirming the findings of the microarray analysis, Hsp70 and Hsp40 were found to be significantly further increased in animals resuscitated with IL-6 when compared to T/HS animals that did not receive IL-6 at resuscitation with 11.2 fold ($p = 0.04$) and 4.5 fold ($p = 0.026$) increases, respectively (Figure 2.)

To assess which of these dysregulated genes may be impacted via IL-6 through Stat3, we incorporated animals pre-treated with a pharmacologic Stat3 inhibitor (GQ T40214) then resuscitated with IL-6 to animal resuscitated with IL-6 alone. Using this combined intergroup approach, we found 12 gene entities with significant dysregulation across all three-group comparisons (Table 3). Interestingly, we find that of the most dysregulated transcripts, the chaperones Hsp70 and Hsp40 demonstrate upregulation in T/HS. In animals in which hepatocyte apoptosis was prevented by receiving IL-6 at resuscitation, we find that Hsp70 and Hsp40 were further upregulated, suggesting a contribution to prevention of hepatocyte apoptosis. When Stat3 is pharmacologically inhibited, however, we find downregulation of these chaperones, suggesting IL-6 acts to upregulate Hsp70/40 via a Stat3-dependent mechanism not previously described.

**T/HS-induced cardiomyocyte apoptosis is prevented by IL-6 resuscitation; the IL-6 effect is mediated, in part, by Stat3.** To confirm our previous findings that T/HS induces cardiomyocyte apoptosis, we measured histone-associated DNA fragments (nucleosomes) in the hearts of rats subjected to our T/HS protocol. Nucleosome levels were significantly increased in comparison to sham in T/HS rats ($p < 0.01$, ANOVA; Table 1). The nucleosome results were confirmed by TUNEL staining with a 12 fold increase in T/HS rats ($p < 0.01$,

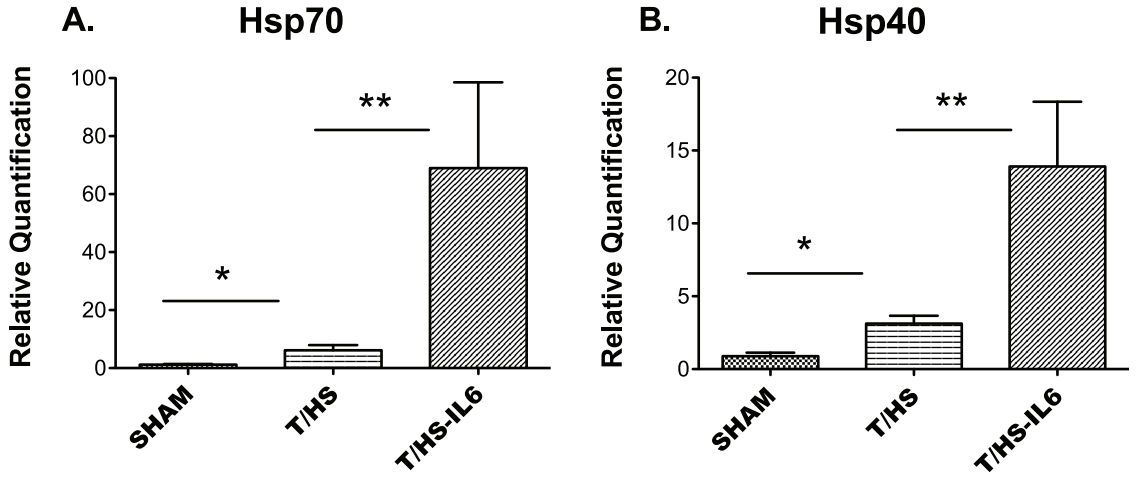



**Figure 2 | Q-RT-PCR using TaqMan® (Life Technologies) for (A) heat shock protein 70 (Hsp70; Hspa1a) and (B) heat shock protein 40 (Hsp40; Dnajb1) performed on whole liver samples from Sham (n = 6), trauma with hemorrhagic shock (T/HS, n = 4), and T/HS animals resuscitated with IL-6 (T/HS-IL6, n = 4).** Transcript values reported as relative quantification (RQ) in comparison to a normal rat liver. Values expressed as mean RQ ± SEM. "*", "**" indicate group comparisons which are statistically different ($p < 0.05$) by T-test.

Table 3 | Liver UPR Transcripts Significantly Altered T/HS vs. Sham, T/HS/IL6 vs. T/HS and T/HS/IL6/GQ Comparisons

| | Regulation | | | Fold Change | | |
|---|---|---|---|---|---|---|
| Gene Symbol | T/HS vs Sham | T/HS-IL6 vs T/HS | T/HS-IL6-GQ vs T/HS-IL6 | T/HS vs Sham | T/HS-IL6 vs T/HS | T/HS-IL6-GQ vs T/HS-IL6 |
| Hspa1a/Hspa1b (Hsp70) | up | up | down | 25.6 | 5.5 | −9.0 |
| Hspa1b (Hsp701b) | up | up | down | 18.4 | 5.9 | −10.8 |
| Ero1l | up | down | down | 9.8 | −3.8 | −1.3 |
| Dnajb1 (Hsp40 subunit) | up | up | down | 5.9 | 2.7 | −3.4 |
| Casp3 (Caspase 3) | up | down | up | 1.8 | −1.7 | 1.4 |
| Sels | up | down | up | 1.5 | −1.3 | 1.4 |
| Eif2ak3 (PERK) | up | down | down | 1.4 | −1.3 | −1.3 |
| Psmb3 | down | up | up | −1.2 | 1.3 | 1.9 |
| Uba1 | down | up | up | −1.3 | 1.2 | 1.8 |
| Psme2 | down | up | up | −1.3 | 1.4 | 2.9 |
| Psme1 | down | up | up | −1.3 | 1.2 | 2.2 |
| Tmbim6 (Bax inhibitor 1) | down | up | down | −1.5 | 1.3 | −1.2 |

ANOVA; Table 1), which also demonstrated that cardiomyocytes represented the overwhelming majority of cells undergoing apoptosis (data not shown).

Nucleosome levels in hearts from IL-6 resuscitated rats were reduced by more than 15 fold compared to placebo treated rats undergoing T/HS ($p < 0.05$, ANOVA). TUNEL assays of sections of rat hearts confirmed these findings with a similar 1.9-fold reduction ($p < 0.05$, ANOVA; Table 1).

Pretreatment of rats with a Stat3 inhibitor was accompanied by a return of nucleosomes to levels similar to those of the placebo treated group. Nucleosome levels in the hearts of T/HS-IL6-GQ rats (Table 1) were increased 6-fold compared to hearts from IL-6 resuscitated rats ($p < 0.05$, ANOVA; Table 1). Thus, pharmacological inhibition of Stat3 using T40214 in rats subjected to severe T/HS resuscitated with IL-6 completely blocked IL-6-mediated prevention of cardiomyocyte apoptosis.

**Heart UPR transcriptome is significantly altered in T/HS.** The results above demonstrate that cardiomyocyte apoptosis caused by T/HS is largely prevented with administration of IL-6 at time of resuscitation (Table 1). We investigated the impact of T/HS on the ER stress response at the transcriptome level, and then defined the role of this ER stress response on the observed reversible cardiomyocyte apoptosis. Using the previously described UPR gene entity list, we found that of the 113 genes present on the chip, 86 (76%) were significantly altered in one-way ANOVA ($p < 0.05$) among all three-group comparisons, T/HS vs. Sham, T/HS-IL6 vs. T/HS, and T/HS-IL6-GQ vs. T/HS-IL6. When the impact of T/HS was looked at specifically, 29 (26%) of those gene entities were significantly dysregulated when compared to sham, with the majority, 79% (23 of 29) significantly upregulated and 6 gene transcripts downregulated. When asking the question of potential mediators of the protective effect of IL-6, 16 entities were significantly altered in both group comparisons (Table 4). The direction of dysregulation induced by T/HS was reversed by IL-6 in all transcripts identified. When taking known apoptotic functions of these genes and the impact of our experimental model into context, we demonstrated that 4 of the 5 genes with known pro-apoptotic function are upregulated following T/HS and subsequently normalized with IL-6 (Table 4). The most dysregulated genes, those genes with $> 2$ fold change, within this intergroup comparison were the chaperones, Hsp70 (10 fold), Hsp40 (3 fold), and Hsp105 (2.5 fold), and the negative regulator of PERK, phosphoinositide-3-kinase interacting protein 1 (Pik3ip1) (−3.0 fold), suggesting, as in the liver, a strong impact on the protein folding mechanics both in the cytoplasm and the endoplasmic reticulum. In contrast to the liver however, the heat

Table 4 | Heart UPR Transcripts Significantly Altered in Both T/HS vs. Sham and T/HS/IL6 vs. T/HS Comparisons

| | | | Regulation | | Fold Change | |
|---|---|---|---|---|---|---|
| Gene Symbol | UPR Function | Apoptosis Function | T/HS vs Sham | IL6 vs T/HS | T/HSvs Sham | IL6 vs T/HS |
| Hspa1a/Hspa1b (Hsp 70) | Chaperone | Anti | up | down | 10 | −6.4 |
| Hspa1b (Hsp701b) | Chaperone | Anti | up | down | 7.5 | −4.9 |
| Cebpb | Transcription factor | Pro | up | down | 5.1 | −1.7 |
| Dnaja1 (Hsp40 subunit) | Co-chaperone | Anti | up | down | 3.1 | −2.3 |
| Hsph1 (Hsp 105) | Chaperone | Anti | up | down | 2.5 | −2.1 |
| Dnajb1 (Hsp40 subunit) | Co-chaperone | Anti | up | down | 2.2 | −2 |
| Nfe2l2 | Transcription factor | Anti | up | down | 2 | −1.4 |
| Ppp1r15a (GADD34) | Transcription factor | Pro | up | down | 2 | −1.4 |
| Xbp1 (X-box-protein 1) | Transcription factor | Pro | up | down | 1.5 | −1.2 |
| Tra1 (Hsp90b1) | Chaperone (ERAD) | Anti | up | down | 1.4 | −1.3 |
| Calr (calreticulin) | Chaperone | Anti | up | down | 1.4 | −1.3 |
| Ddit3 (CHOP) | Transcription factor | Pro | up | down | 1.4 | −1.3 |
| Serp1 | protects unfolded proteins from ERAD | Anti | up | down | 1.3 | −1.2 |
| Sp1 | Transcription factor | Pro | down | up | −1.2 | 1.3 |
| Sels | modulates ATF6 | Unknown | down | up | −1.5 | 1.3 |
| Pik3ip1 | negative regulator of PERK | Anti | down | up | −3 | 1.4 |

Abbreviation: ERAD, endoplasmic reticulum-associated degradation.

shock protein chaperones, Hsp70 and Hsp40, were downregulated in the hearts of IL-6-treated animals, indicating they likely are not contributing to the apoptotic protection conferred by IL-6. When adding the comparison of GQ T40214 to IL-6 group, we found 11 gene entities with significant dysregulation across all three group comparisons, and, of those, 8 suggest potential IL-6 mediated effect through Stat3 (Table 5).

## Discussion

Our findings provide the first-ever global description of the UPR transcriptome of the heart and liver following T/HS. We demonstrated that T/HS leads to significant cardiomyocyte and hepatocyte apoptosis, which is prevented through the Stat3-dependent actions of IL-6. We examined the UPR transcriptome to identify candidate gene transcripts responsible for T/HS-induced apoptosis. By utilizing an expanded repertoire of UPR members, both canonical and non-canonical, and the reproducible and measurable outcome of IL-6-preventable apoptosis in our model of T/HS, we were able to identify potential UPR modulators that significantly impact T/HS-induced hepatocyte and cardiomyocyte apoptosis.

In the liver, members of the heat shock family of protein folding chaperones, Hsp70 and Hsp40, emerged as significant potential non-canonical UPR modulators of hepatocyte apoptosis in our model of T/HS. This compares with findings in other models of organ injury, such as work done by Wang et al., which demonstrated that Hsp70 and its induction with geranylgeranylacetone (GGA) can protect against primary proximal tubule apoptosis and acute kidney damage in an ischemic injury model[27], and work done by Kuboki et al., which demonstrated in a partial liver I/R model that induction of Hsp70 with sodium arsenite reduced liver injury, as determined by transaminase levels and histology[28]. Besides their role in protein folding in the cytoplasmic space, heat shock proteins have been linked to the canonical UPR pathways of the endoplasmic reticulum. One example is Hsp72, a Hsp 70 family member, which has been shown to interact with the cytosolic domain of IRE1α, enhancing XBP1 splicing, and attenuating apoptosis *in vitro*[29]. Heat shock protein chaperones have also been shown to prevent CHOP-induced apoptosis through the Hsp70-DnaJ chaperone pair inhibiting translocation of Bax to mitochondria *in vitro*[30].

Our findings provide *in vivo* data linking the heat shock protein family to hepatocyte apoptosis possibly via a Stat3-dependent mechanism in T/HS. These findings are supported by previous work linking IL-6/Stat3 transcriptional regulation of heat shock protein family members[31]. Hsp70 and Hsp40 appear to contribute to an adaptive and protective process in the liver, demonstrating upregulation in T/HS and further upregulation in livers of IL-6-resuscitated animals, correlating with prevention of apoptosis. However, when animals were pretreated with a Stat3 inhibitor that blocked IL-6's prevention of apoptosis, these chaperone transcripts were downregulated. Thus, these findings suggest that IL-6, via a Stat3-dependent pathway, acts to superinduce Hsp70 and 40 transcripts in T/HS. These findings are supported by the work of Masumichi et al[32], which demonstrate that IL-6 is necessary for upregulation of heat shock protein members, including Hsp70/40, in a model of acetaminophen-induced hepatic injury.

Interestingly, the canonical members of the UPR, while altered, were not the most dysregulated transcripts in the liver. CCAAT/-enhancer-binding protein homologous protein (CHOP), PERK, alpha subunit of eukaryotic initiation factor 2 (Eif2α), activating transcription factor 4 (ATF4), and calreticulin were significantly dysregulated (6 to −1.2 fold change) in T/HS (Supplemental Table 1). When considering those entities altered > 2-fold and taking into account known UPR and apoptotic functions of the canonical UPR members, only the transcriptional profile of ATF4 suggested a maladaptive contribution to hepatocyte apoptosis. However, this maladaptive role does not appear to be mediated through Stat3.

The heart demonstrated a distinctly different UPR transcriptional profile in comparison to the liver. When one considers the nature and functions of these organs, this is not unexpected. The liver is the largest glandular mass of tissue in the body and is highly secretory with both exocrine and endocrine function, whereas the heart, with myocyte predominance, is largely non-secretory with maintenance of biophysical function more paramount. The impact of T/HS on the canonical UPR transcriptome was even less in magnitude in the heart than in the liver. Significantly dysregulated canonical UPR transcripts included CHOP, PERK, X-box binding protein 1 (XBP1), Eif2α, and calreticulin (2.5 to −1.2 fold change) with only ATF4 dysregulated by more than 2 fold in response to T/HS (Supplemental Table 2). When taking into account known UPR and apoptotic function, CHOP, XBP1, and GADD34 exhibit transcriptional profiles suggestive of an adaptive role in T/HS-induced cardiomyocyte apoptosis. Given the fold-change was nominal (1.4 to 2-fold) however, further investigation is required to determine their true contribution to T/HS-induced apoptosis.

The protein folding chaperones, Hsp70 and Hsp40, which proved important modulators of apoptosis in the liver, were upregulated in the heart following T/HS, but were downregulated in animals in which IL-6 prevented cardiomyocyte apoptosis, suggesting these chaperones may play a maladaptive role in T/HS-induced cardiomyocyte apoptosis. The dichotomous nature of these chaperones' roles in the liver and heart in T/HS is supported by work in other models of organ injury. Indeed, previous studies have suggested that Hsp70 family proteins may serve to augment cardiac inflammation and contractile dysfunction[33,34], and its downregulation in IL-6

Table 5 | Heart UPR Transcripts Significantly Altered Across T/HS vs. Sham, T/HS/IL6 vs. T/HS, and T/HS/IL6/GQ vs. T/HS/IL6 Comparisons

| | Regulation | | | Fold Change | | |
|---|---|---|---|---|---|---|
| Gene Symbol | T/HS vs Sham | T/HS-IL6 vs T/HS | T/HS-IL6-GQ vs T/HS-IL6 | T/HS vs Sham | T/HS-IL6 vs T/HS | T/HS-IL6-GQ vs T/HS-IL6 |
| Dnaja1 (Hsp40 subunit) | up | down | up | 3.1 | −2.3 | 2.0 |
| Hsph1 (Hsp105) | up | down | up | 2.5 | −2.1 | 1.5 |
| Nfe2l2 | up | down | up | 2.0 | −1.4 | 2.0 |
| Ppp1r15a (GADD34) | up | down | up | 2.0 | −1.4 | 1.4 |
| Xbp1 (X-box-protein 1) | up | down | up | 1.5 | −1.2 | 1.8 |
| Tra1 | up | down | up | 1.4 | −1.3 | 1.2 |
| Calr (calreticulin) | up | down | up | 1.4 | −1.3 | 1.4 |
| Ddit3 (CHOP) | up | down | up | 1.4 | −1.3 | 5.5 |
| Sp1 | down | up | up | −1.2 | 1.3 | 1.6 |
| Sels | down | up | up | −1.5 | 1.3 | 3.1 |
| Pik3ip1 | down | up | up | −3.0 | 1.4 | 1.8 |

treated animals would support this hypothesis, as we have previously demonstrated that IL-6 acts to preserve contractile function following T/HS[3]. However, Yao et al., have recently shown that Hsp70 upregulation may contribute to the cardioprotection against ischemia/reperfusion injury observed with lipopolysaccharide (LPS) pretreatment[35]. Thus, the role of Hsp70 in myocardial ischemia/reperfusion injury may be specific to the insult and requires further study to clarify the adaptive versus maladaptive role it may play in ischemia/reperfusion events such as resuscitated hemorrhagic shock.

In addition to providing a global description of the UPR transcriptome of the heart and liver following T/HS, our findings demonstrate that IL-6, when utilized as a resuscitation adjuvant, may augment a physiologic protective role of Hsp70 and Hsp40 via a Stat3-dependent mechanism, thereby protecting against hepatocyte apoptosis. These findings support the concept that modulators of Hsp70 or 40 may offer a therapeutic strategy for prevention of apoptosis and ultimately hepatic dysfunction following T/HS.

## Methods

**Rat T/HS protocol.** For the rat experiments in this study, 8-week old male Sprague-Dawley rats (200–250 gm) were used. Rats were subjected to the sham or T/HS protocols, as described[3,6,23] with modifications. Blood was withdrawn into a heparinized syringe to achieve and then maintain the target MAP at 35 mmHg until blood pressure compensation failed. Blood was then returned as needed to maintain the target MAP. The amount of shed blood returned (SBR) defined shock severity as reflected in the duration of hypotension, and the animals used in this analysis received 50% SBR (SBR50; duration of hypotension, 273 ± 24.9 minutes). At the end of the hypotensive period, rats were resuscitated as described[3,6,23] and humanely sacrificed 60 minutes after the start of resuscitation in order to capture the first wave of transcriptional changes. Where indicated, rats received 10 μg/kg of recombinant human IL-6 in 0.1 ml PBS at the initiation of the resuscitation or PBS alone. Sham rats were anesthetized and cannulated for 250 minutes but were not subjected to hemorrhage or resuscitation. Rat livers and hearts were harvested immediately after sacrifice and snap frozen in liquid nitrogen for nucleosome and RNA extraction steps.

**In vivo pharmacological inhibition of Stat3.** To achieve pharmacological inhibition of Stat3 activity within the rats, the G-rich, quartet-forming oligodeoxynucleotides (GQ-ODN), T40214[24] (2.5 mg ODN/kg) was given by tail vein injection, 24 hours prior to subjecting them to the SBR50 protocol with IL-6 treatment. The half-life of T40214 in tissues is ≥ 48 hours[25].

**Nucleosome ELISA.** Levels of histone-associated DNA fragments (nucleosomes) were determined in homogenates of snap-frozen liver using an ELISA method (Cell Death Detection ELISA$^{plus}$; Roche Diagnostics, Manheim, Germany), as described[6,23]. The nucleosome concentration for each liver sample was normalized for total protein concentration determined by Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA). The final nucleosome concentration for each liver sample was the average of duplicate determinations.

**Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining.** TUNEL staining to enzymatically detect the free 3′-OH DNA termini was performed using the ApopTag Plus Peroxidase in situ Apoptosis Detection Kit from Chemicon International (now Millipore, Billerica, MA). Slides were rehydrated from xylene to PBS through a series of decreasing concentrations of ethanol and digested in proteinase K (20 μg/ml) for 3 minutes at 23°C. Endogenous peroxidases were quenched for 30 minutes in 3% hydrogen peroxide in PBS. TdT enzyme was diluted in TUNEL solution buffer then used as suggested by the manufacturer. Slides were counterstained with hematoxylin. TUNEL positive cells were assessed microscopically by counting the total nuclei and the number of TUNEL-positive nuclei in twenty random 1000× fields by an experienced histologist, blinded to the treatment each rat received. Data is presented as the number of TUNEL positive cells per high power field (hpf).

**RNA isolation and oligonucleotide microarray hybridization.** Total RNA was isolated from 4–5 micron cryotome sections of liver using TRIzol® Reagent (Invitrogen, Carlsbad, California) single step RNA isolation protocol followed by purification with RNeasy® Mini Kit (QIAGEN, Hilden, Germany) as instructed by the manufacturer. Gene expression profiling was performed with the Affymetrix Rat Array RAE 230A chips following Affymetrix protocols used within the Baylor College of Medicine Microarray Core Facility.

**Microarray analysis.** We used GenespringGX (Agilent Technologies Inc., Santa Clara CA) software package for quality assessment, statistical analysis and annotation.

Low-level analyses included background correction, quartile normalization and expression estimation using RMA-based analysis within Genespring. One-way analysis of variance (ANOVA) with contrasts was used for group comparisons on all genes and on the list of UPR entities. P-values were adjusted for multiple comparisons using the Benjamini-Hockberg method. The adjusted p-values represent false discovery rates (FDR) and are estimates of the proportion of "significant" genes that are false or spurious "discoveries". We used a FDR = 5% as cut-off. The genechip used, RAE 230A, contained 15,923 probe sets representing 13,521 annotated genes or expressed sequence tags. A UPR gene entity list was created using both Ingenuity Pathway Analysis (IPA® Redwood City, CA) and the Gene Ontology Database©, with keywords "endoplasmic reticulum stress, unfolded protein response". Three or more chips for each organ were hybridized using mRNA isolated from hearts and livers, respectively for each group comparison: Sham (4), T/HS-PBS (4) and T/HS-IL6 (4) and T/HS-IL6-GQ (3) groups.

**Quantitative (Q) RT-PCR.** Two-step Q-RT-PCR was performed using the ABI Prism 7700 sequence detection system (Perkin-Elmer/Applied Biosystems, Foster City, CA) as described previously[3,26]. Briefly, total RNA (1 μg) was reverse transcribed using reverse transcription reagents (BioRad catalog no. 170-8842; Hercules, CA); 20% of each RT reaction was used in duplicate PCR reactions using TaqMan® Universal Master Mix II, with uracil N-glycosylase (PN 4440038) and specific primer and probe sets designed by the manufacturer (TaqMan Gene Expression Assay, Applied Biosystems, Darmstadt, Germany)—Hsp70 (Hspa1a; catalog no. Rn04224718_u1), Hsp40 (Dnajb1; catalog no. Rn 01426952_g1), and 18S rRNA (catalog no.Rn03928990_g1). Each PCR amplification run consisted of incubation for 5 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The cycle threshold of each duplicate determination was normalized by subtraction of the cycle threshold for its corresponding 18S rRNA ($\Delta C_T$). Each $\Delta C_T$ was then calibrated by subtracting the $\Delta C_T$ value for control rat tissue ($\Delta\Delta C_T$). RNA amount was expressed as relative units calculated as $2^{-\Delta\Delta CT}$, as described[26].

**Statistical analysis.** Statistical differences between experimental groups were analyzed using one-way ANOVA and post-hoc analysis was performed using Student-Newman-Keuls test. T-test analysis performed using unpaired Student's T-test.


1. Minino, A., Anderson, R., Fingerhut, L., Boudreault, M. & Warner, M. Deaths: injuries, 2002. *Natl Vital Stat Rep* **54**, 1–124 (2006).
2. Dewar, D., Moore, F. A., Moore, E. E. & Balogh, Z. Postinjury multiple organ failure. *Injury* **40**, 912–918 (2009).
3. Alten, J. *et al.* Prevention of hypovolemic circulatory collapse by IL-6 activated Stat3. *PLoS One* **3**, e1605 (2008).
4. Guan, J., Jin, D.-D., Jin, L.-J. & Lu, Q. Apoptosis in organs of rats in early stage after polytrauma combined with shock. *J Trauma* **52**, 104–111 (2002).
5. Jaskille, A. *et al.* Hepatic apoptosis after hemorrhagic shock in rats can be reduced through modifications of conventional Ringer&'s solution. *Journal of the American College of Surgeons* **202**, 25–35 (2006).
6. Moran, A. *et al.* Prevention of trauma and hemorrhagic shock-mediated liver apoptosis by activation of stat3alpha. *Int J Clin Exp Med* **1**, 213–247 (2008).
7. Sundar, S. V., Li, Y.-Y., Rollwagen, F. M. & Maheshwari, R. K. Hemorrhagic shock induces differential gene expression and apoptosis in mouse liver. *Biochemical and Biophysical Research Communications* **332**, 688–696 (2005).
8. Cox, J. S., Shamu, C. E. & Walter, P. Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* **73**, 1197–1206 (1993).
9. Mori, K., Ma, W., Gething, M. J. & Sambrook, J. A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus. *Cell* **74**, 743–756 (1993).
10. Normington, K., Kohno, K., Kozutsumi, Y., Gething, M. J. & Sambrook, J. S. cerevisiae encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell* **57**, 1223–1236 (1989).
11. Shamu, C. E. & Walter, P. Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *The EMBO journal* **15**, 3028–3039 (1996).
12. Dara, L., Ji, C. & Kaplowitz, N. The contribution of endoplasmic reticulum stress to liver diseases. *Hepatology (Baltimore, Md)* **53**, 1752–1763 (2011).
13. Lawless, M. W. *et al.* Activation of endoplasmic reticulum-specific stress responses associated with the conformational disease Z alpha 1-antitrypsin deficiency. *Journal of immunology (Baltimore, Md: 1950)* **172**, 5722–5726 (2004).
14. Malhi, H. & Kaufman, R. J. Endoplasmic reticulum stress in liver disease. *Journal of hepatology* **54**, 795–809 (2011).
15. Du, K., Herzig, S., Kulkarni, R. N. & Montminy, M. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science* **300**, 1574–1577 (2003).
16. Ota, T., Gayet, C. & Ginsberg, H. N. Inhibition of apolipoprotein B100 secretion by lipid-induced hepatic endoplasmic reticulum stress in rodents. *J Clin Invest* **118**, 316–332 (2008).
17. Bailly-Maitre, B. *et al.* Cytoprotective gene bi-1 is required for intrinsic protection from endoplasmic reticulum stress and ischemia-reperfusion injury. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 2809–2814 (2006).
18. Sakon, M., Ariyoshi, H., Umeshita, K. & Monden, M. Ischemia-reperfusion injury of the liver with special reference to calcium-dependent mechanisms. *Surgery today* **32**, 1–12 (2002).

19. Duvigneau, J. C. *et al*. Reperfusion does not induce oxidative stress but sustained endoplasmic reticulum stress in livers of rats subjected to traumatic-hemorrhagic shock. *Shock (Augusta, Ga)* **33**, 289–298 (2010).
20. Jian, B. *et al*. Activation of endoplasmic reticulum stress response following trauma-hemorrhage. *Biochim Biophys Acta* **1782**, 621–626 (2008).
21. Doroudgar, S., Thuerauf, D. J., Marcinko, M. C., Belmont, P. J. & Glembotski, C. C. Ischemia activates the ATF6 branch of the endoplasmic reticulum stress response. *J Biol Chem* **284**, 29735–29745 (2009).
22. Fu, H. Y. *et al*. Ablation of C/EBP homologous protein attenuates endoplasmic reticulum-mediated apoptosis and cardiac dysfunction induced by pressure overload. *Circulation* **122**, 361–369 (2010).
23. Moran, A. *et al*. IL-6-Mediated Activation of Stat3α Prevents Trauma/Hemorrhagic Shock-Induced Liver Inflammation. *PLoS One* **6**, e21449 (2011).
24. Jing, N. *et al*. G-quartet oligonucleotides: a new class of signal transducer and activator of transcription 3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis. *Cancer Res* **64**, 6603–6609 (2004).
25. Jing, N., Sha, W., Li, Y., Xiong, W. & Tweardy, D. J. Rational drug design of G-quartet DNA as anti-cancer agents. *Curr Pharm Des* **11**, 2841–2854 (2005).
26. Ono, M., Yu, B., Hardison, E. G., Mastrangelo, M.-A. A. & Tweardy, D. J. Increased susceptibility to liver injury after hemorrhagic shock in rats chronically fed ethanol: role of nuclear factor-kappa B, interleukin-6, and granulocyte colony-stimulating factor. *Shock (Augusta, Ga)* **21**, 519–525 (2004).
27. Wang, Z. *et al*. Induction of heat shock protein 70 inhibits ischemic renal injury. *Kidney Int* **79**, 861–870 (2011).
28. Kuboki, S. *et al*. Role of heat shock protein 70 in hepatic ischemia-reperfusion injury in mice. *Am J Physiol Gastrointest Liver Physiol* **292**, G1141–9 (2007).
29. Gupta, S. *et al*. HSP72 protects cells from ER stress-induced apoptosis via enhancement of IRE1alpha-XBP1 signaling through a physical interaction. *PLoS Biol* **8**, e1000410 (2010).
30. Gotoh, T., Terada, K., Oyadomari, S. & Mori, M. hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. *Cell Death Differ* **11**, 390–402 (2004).
31. Stephanou, A. & Latchman, D. S. Transcriptional regulation of the heat shock protein genes by STAT family transcription factors. *Gene expression* **7**, 311–319 (1999).
32. Masubuchi, Y. *et al*. Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease. *Biochemical and Biophysical Research Communications* **304**, 207–212 (2003).
33. Mathur, S., Walley, K. R., Wang, Y., Indrambarya, T. & Boyd, J. H. Extracellular Heat Shock Protein 70 Induces Cardiomyocyte Inflammation and Contractile Dysfunction via TLR2. *Circulation journal : official journal of the Japanese Circulation Society* (2011).
34. Zou, N. *et al*. Critical role of extracellular heat shock cognate protein 70 in the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion. *Am J Physiol Heart Circ Physiol* **294**, H2805–13 (2008).
35. Yao, Y.-W. *et al*. Lipopolysaccharide pretreatment protects against ischemia/reperfusion injury via increase of HSP70 and inhibition of NF-κB. *Cell Stress & Chaperones* **16**, 287–296 (2011).

## Acknowledgements

Supported, in part, by grant R01 HL07619 (DJT) from the National Heart, Lung and Blood Institute of the National Institutes of Health, grant DM090899 (DJT) from the Department of Defense, and grant T32 AI055413 (SAT) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

## Author contributions

S.T. and D.T. contributed to experimental design. S.T. and A.A. performed animal hemorrhagic shock protocols. S.T., P.R. and D.T. contributed to all tables. S.T. and D.T. contributed to figure 1. S.T., D.T. and P.R. contributed to figure 2, S.T. and D.T. wrote the main manuscript text. All authors reviewed the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.



**How to cite this article:** Thacker, S.A., Robinson, P., Abel, A. & Tweardy, D.J. Modulation of the Unfolded Protein Response During Hepatocyte and Cardiomyocyte Apoptosis In Trauma/Hemorrhagic Shock. *Sci. Rep.* **3**, 1187; DOI:10.1038/srep01187 (2013).

Journal of Infection (2013) 66, 87—94

BIA
British Infection Association

www.elsevierhealth.com/journals/jinf

# Increased apoptosis of peripheral blood neutrophils is associated with reduced incidence of infection in trauma patients with hemorrhagic shock

C. Anne Morrison [a], Ana Moran [b], Shruti Patel [b], Maria del Pilar Huby Vidaurre [b], Matthew M. Carrick [a], David J. Tweardy [b,*]

[a] *Michael E. DeBakey Department of Surgery, Section of Infectious Disease, Baylor College of Medicine, One Baylor Plaza, N-1318, Houston, TX 77007, USA*
[b] *Department of Medicine, Section of Infectious Disease, Baylor College of Medicine, Houston, TX, USA*

Accepted 3 October 2012
Available online 9 October 2012

**KEYWORDS**
Apoptosis;
Neutrophil;
Infection;
Trauma;
Shock;
Hemorrhage

**Summary** *Objective:* We aimed to describe the relationship between early peripheral leukocyte apoptosis and incidence of subsequent infection in trauma patients with hemorrhagic shock (T/HS).
*Methods:* T/HS patients requiring emergency surgery were prospectively enrolled. Nucleosome ELISA and TUNEL staining were performed on peripheral blood drawn pre-operatively, post-operatively and at 24 h. Subjects were followed for 30 days or until death or hospital discharge to record all episodes of infection.
*Results:* Forty-one subjects were enrolled. Six died within 24 h of surgery and were not included in the analysis. Nucleosome levels peaked post-operatively and dropped to baseline levels at 24 h ($p = 0.03$). TUNEL analysis revealed that polymorphonuclear neutrophils (PMNs) accounted for 72% of apoptotic leukocytes; the remaining apoptotic cells were mainly lymphocytes. Increased post-operative leukocyte apoptosis was associated with decreased systemic inflammatory response syndrome (SIRS) severity. Seventeen of the 35 survivors (48.6%) developed infections, while 18 (51.4%) did not. Pre-operative and post-operative nucleosome levels were 2.5 and 3 times higher, respectively, in T/HS patients who did not develop infection compared to those who did. Increased nucleosome levels were associated in particular with protection against sepsis ($p=0.03$) and multiple infections ($p = 0.01$).
*Conclusion:* Peripheral blood PMN apoptosis in the early resuscitative period is associated with decreased incidence of subsequent infection in T/HS patients.


* Corresponding author. Tel.: +1 713 798 8908; fax: +1 713 798 8948.
*E-mail addresses:* camorris@bcm.edu (C. Anne Morrison), amoran@bcm.edu (A. Moran), shrutigpatel@gmail.com (S. Patel), huby@bcm.edu (M.delP.H. Vidaurre), mcarrick@bcm.edu (M.M. Carrick), dtweardy@bcm.edu (D.J. Tweardy).

0163-4453/$36 
http://dx.doi.org/10.1016/j.jinf.2012.10.001

## Introduction

Trauma continues to be an enormous public health problem and is a leading cause of death around the world.[1] In the United States, trauma is the fifth leading cause of death overall and the number one cause of death for individuals between the ages of 1 and 45 years.[2] With over 170,000 trauma deaths in the US in 2005, more Americans died of injuries than from cancers of the breast, colon, prostate, liver and pancreas combined.[3] Historically, the majority of trauma victims die before reaching the hospital[4]; however, more recent epidemiologic studies have shown that advances in trauma care systems and emergency medical services have resulted in a significantly larger percentage of patients who survive to hospital admission.[5] Although exsanguination and head injury continue to account for a large proportion of early trauma deaths, the majority of late trauma deaths occur as a result of infection and/or multiple organ failure (MOF).[5]

The clinical association between late trauma deaths and the development of MOF and infections has been well established since the 1970s.[6] However, it has primarily been in the past two decades or so that researchers have focused their investigations on the body's immunological and inflammatory responses to trauma-resuscitation at a molecular and cellular level in an effort to better understand MOF in this setting and how it predisposes to infections.

In addition to playing a key role in protection against acute bacterial infections, polymorphonuclear neutrophils (PMNs) modulate the innate immune response to non-infectious tissue insults such as trauma and shock. PMN apoptosis has been shown to be blunted in patients with SIRS[7] and sepsis[8,9] where it is thought to exacerbate tissue injury.[10] Reduced PMN apoptosis has been attributed, at least in part, to production of pro-inflammatory cytokines such as G-CSF that prolong PMN survival.[11–13] Further studies are needed to improve our understanding of the impact of altered PMN apoptosis on outcomes of trauma, particularly infections that complicate MOF.

We previously demonstrated in a rat model of trauma and hemorrhagic shock that cardiac,[14] hepatocyte,[15] alveolar epithelial cells[16] and PMN apoptosis (Tweardy et al., 2006, unpublished findings) are increased as a function of the duration of hypotensive phase and that apoptosis peaks approximately 4 h following initiation of resuscitation. In the current study, we examined the hypotheses that increased peripheral PMN apoptosis occurs early in the resuscitative period in trauma/hemorrhagic shock (T/HS) patients and is associated with a decreased incidence of infections. Our findings show that peripheral leukocyte apoptosis occurs in T/HS patients, achieves maximum levels immediately post-operatively and normalizes by 24 h. PMN were the predominant cell within the leukocyte population undergoing apoptosis. In addition, the degree of leukocyte apoptosis was inversely proportional to systemic inflammatory response syndrome (SIRS) severity and the risk of infection. These findings support the hypothesis that neutrophil apoptosis limits early tissue injury thereby decreasing subsequent susceptibility to infection in hospitalized T/HS patients.

## Material and methods

### Patient description and enrollment

This research was conducted at Ben Taub General Hospital, a level-one trauma center located in Houston, TX. Trauma patients arriving consecutively at the Emergency Center (EC) with a systolic blood pressure less than 90 mm Hg who required emergent laparotomy or thoracotomy were enrolled and brought immediately to the operating room (OR) where they underwent simultaneous fluid resuscitation and repair of their injuries. All samples and outcomes data were collected prospectively as part of a separate, ongoing randomized controlled clinical trial at our institution comparing hypotensive resuscitation to standard fluid resuscitation for T/HS patients.[17] Inclusion criteria for the study included traumatic injury to the chest and/or abdomen requiring emergent laparotomy or thoracotomy and at least one documented systolic blood pressure less than 90 mm Hg. Exclusion criteria included any of the following: (1) age $>$45 years or $<$14 years; (2) pregnancy; (3) incarceration; (4) known history of coronary artery disease, renal disease or cerebrovascular disease; (5) patients in whom traumatic brain injury could not be definitively ruled out based upon mechanism of injury and/or negative CT scan of the head. Patients enrolled in the clinical trial that fulfilled all the inclusion criteria and did not fulfill any exclusion criterion were enrolled in the study. In just over half the cases, emergent surgery was initiated before collection of the pre-operative blood sample could be done. Such patients were excluded from the study. Patients were followed daily for 30 days or until death or hospital discharge. Patients' vital signs and all incidences of infection were recorded.

### Venous blood sampling and leukocyte apoptosis studies

Peripheral venous blood samples were drawn at three time points: pre-operatively, post-operatively, and at 24 h. Pre-operative samples were collected either in the EC or in the OR immediately preceding the start of the case; post-operative samples were drawn upon transfer from the OR to the surgical intensive care unit (SICU); and 24-h samples were drawn at 24 h after admission to the SICU. Timing of the immediate post-operative blood sample was based upon our previous finding that peak levels of cell apoptosis typically occur within 4 h of initiating fluid resuscitation following hemorrhagic shock in animals.[14–16] Five milliliters of blood was collected in two heparinized tubes. Peripheral blood leukocytes were harvested by dextran sedimentation, as previously described,[18] and the cell pellets immediately frozen for protein extraction. Protein was extracted using lysis buffer (Roche) and quantified using the Bradford method. Nucleosome ELISA was then performed using the Cell Death Detection ELISAplus® kit (Roche). In addition to the nucleosome ELISA assay, cytospins were prepared from leukocytes isolated from 8 of the post-operative blood samples and TUNEL stained as described.[14] The percentage of TUNEL-positive leukocytes, polymorphonuclear leukocytes (PMN) and mononuclear cells within each

sample was enumerated microscopically within 20 random 1000× fields by one of the authors (AM) experienced in blood cell histology.

### Clinical data

Vital signs (heart rate, temperature, respiratory rate and blood pressure) were recorded for the entire study duration of 30 days or till discharge or death. Clinical outcomes of mortality and infection were also recorded. Infection was defined according to the criteria described in Table 1. Patient had to exhibit all the listed criteria in order to be diagnosed with infection. Culture positivity was included as one of the criteria for more stringent and accurate diagnosis. The association of peak post-operative nucleosome level with SIRS data collected at three definitive time points, namely pre-operative, post-operative and 24 h was analyzed for each participating patient in the study.

### Statistical analysis

Statistical analysis was performed using STATA® statistical software package, version 10.0 (StataCorp, College Station, TX). Comparisons of continuous, independent variables were performed using the Wilcoxon–Mann–Whitney test. Comparisons of continuous, paired variables were performed using the Wilcoxon Signed Rank test.

## Results

### Peripheral blood leukocyte apoptosis peaks immediately post-operatively and consists mostly of neutrophils

Forty-one patients had post-operative blood samples drawn and were included in the study. The subjects' baseline characteristics and intra-operative fluid requirements are shown in Table 2. The high prevalence of racial minorities noted in this study roughly reflects the racial breakdown of penetrating trauma victims treated at our urban, county hospital. The mean time from presentation to the emergency center to arrival in the OR was 15.6 min. The mean duration of surgery was 114 min.

Nucleosome levels in study subjects peaked at the post-operative time point, and then dropped to pre-operative levels at 24 h (Fig. 1A). The 41% decrease of nucleosome levels at 24-h vs. the post-operative time point was statistically significant ($p = 0.03$); the 1.7-fold increase in post-operative nucleosome levels vs. pre-operative levels nearly reached statistical significance ($p = 0.06$).

TUNEL analysis was performed on eight consecutive post-operative peripheral blood samples in order to quantify the percent of leukocytes undergoing apoptosis and to identify the type of leukocytes undergoing apoptosis (polymorphonuclear neutrophils [PMNs] vs. mononuclear cells). Overall, 9% of all peripheral leukocytes were apoptotic; PMNs accounted for 72% of the apoptotic cells with remaining cells consisting of lymphocytes.

**Table 1** Types of infections and criteria for their diagnosis.

| |
|---|
| Pneumonia |
| 1. Infiltrate on chest X-ray |
| 2. Positive sputum culture |
| 3. Fever and/or leukocytosis |
| Intra-abdominal infection |
| 1. Fluid collection requiring drainage |
| 2. Fluid described as purulent |
| 3. ± Positive culture |
| Bacteremia and sepsis |
| 1. Positive blood cultures |
| 2. Meets ≥2 SIRS criteria |
| Urinary tract infection |
| 1. Positive urine culture and/or urinalysis |
| 2. Fever and/or leukocytosis |
| Wound infection |
| 1. Erythema and/or wound drainage |
| 2. Fever and/or leukocytosis |
| 3. ± Positive wound culture |

Patients must exhibit all of the criteria listed for each diagnosis.

### Peripheral blood leukocyte apoptosis is inversely correlated with SIRS criteria during early resuscitation

Using a linear regression model, we found that post-operative nucleosome levels were inversely correlated to heart rate at 24 h (Fig. 1B; correlation coefficient $r = -0.36$, $p = 0.02$) and at 48 h (Fig. 1C; $r = -0.39$, $p = 0.01$). Post-operative nucleosome levels also were inversely correlated to temperature at 48 h (Fig. 1D; $r = -0.38$, $p = 0.01$). No such correlations were seen when comparing pre-operative or 24-h nucleosome levels to heart rate or temperature. Respiratory rate was not included in this analysis since the vast majority of patients were sedated and intubated and many of these patients had no spontaneous respirations over the ventilator settings.

### Higher peripheral blood PMN apoptosis in the early resuscitative period is associated with incidence of subsequent infection

Of the 41 subjects in whom post-operative samples were obtained, six died within 24 h of surgery. Of the 35 survivors, 17 (48.6%) developed an infection over the next 30 days, three of whom subsequently died. Infections were defined as listed in Table 1; the incidence of each type of infection is listed in Table 3. Nine of the 17 subjects who developed an infection had multiple (≥2) infections. The mean number of infections in those who developed any infection was 1.8 (range 1–5). Eighteen subjects who survived past the initial 48 h never developed any type of infection during the next 30 days. Of these patients, one subsequently died.

**Table 2** Patient characteristics ($n = 41$).

| Demographics | | | |
|---|---|---|---|
| | Infection | No infection | Total |
| Age (mean ± SD) | 31.6 ± 10.1 | 33.8 ± 8.6 | 32.5 ± 9.3 |
| % Male | 92% | 94% | 93% |
| Black | 47% | 54% | 51% |
| Hispanic | 53% | 42% | 46% |
| Asian | 0% | 4% | 2% |
| Mechanism | | | |
| Blunt trauma | 0% | 4% | 2% |
| Gunshot wound | 94% | 63% | 76% |
| Stab wound | 6% | 33% | 22% |
| Presenting vital signs mean ± SD | | | |
| Systolic BP | 80 ± 20 | 71.9 ± 26.8 | 75 ± 24 |
| Diastolic BP | 49 ± 20 | 34.7 ± 16.9 | 40 ± 19 |
| Pulse | 113 ± 18 | 98.7 ± 43 | 104 ± 36 |
| Baseline labs mean ± SD | | | |
| Base deficit | −9.2 ± 4.7 | −13.8 ± 7.7 | −12.0 ± 7.0 |
| Hemoglobin | 29.9 ± 4.3 | 31.8 ± 7.1 | 31.1 ± 6.2 |
| Glucose | 201.8 ± 58.5 | 223 ± 99 | 214 ± 84 |
| Injury severity score mean ± SD | | | |
| RTS[a] | 10.2 ± 1.8 | 9.8 ± 6.2 | 10 ± 4.6 |
| ISS[b] | 23.8 ± 10.8 | 20.7 ± 12.9 | 22.0 ± 12 |
| TRISS[c] | 0.97 ± 0.02 | 0.81 ± 0.33 | 0.88 ± 0.27 |
| IV fluids mean ± SD | | | |
| Crystalloid (mL) | 3588 ± 2039 | 3350 ± 1861 | 3449 ± 1915 |
| Colloid (mL) | 912 ± 404 | 458 ± 405 | 646 ± 464 |
| Transfusions mean ± SD | | | |
| PRBC's (mL) | 1515 ± 1937 | 2521 ± 2725 | 2100 ± 2400 |
| FFP (mL) | 383 ± 559 | 516 ± 943 | 460 ± 800 |
| Platelets | 50 ± 140 | 118 ± 943 | 100 ± 210 |
| Total transfusions | 1985 ± 2419 | 3115 ± 3696 | 2660 ± 3250 |
| Total inputs | 6456 ± 3803 | 6963 ± 4640 | 6750 ± 4270 |

[a] Revised trauma score.
[b] Injury severity score.
[c] Trauma-injury severity score.

There were no statistically significant differences at baseline between those who developed an infection vs. those who did not develop any infection with regards to each of the characteristics shown in Table 2 ($p > 0.05$ for all comparisons), with the exception of colloid administration. Patients who developed infection received more colloid intra-operatively than those who did not develop infection (910 ml vs. 530 ml; $p = 0.01$). There was no significant difference between the infected and uninfected patient groups regarding pre or peri-operative antibiotic use. Also there was no significant association between the risk of developing infection and randomization group for the trial determining the impact of hypotensive resuscitation vs. standard fluid resuscitation.

Of note, subjects who did not develop any infection had 3-fold higher leukocyte nucleosome levels pre-operatively (40.2 mU/mg; Fig. 2) than those who did develop an infection (13.6 mU/mg; $p = 0.04$). In addition, those who did not develop infection had 2.5-fold higher leukocyte apoptosis level post-operatively (49.8 mU/mg; Fig. 2 and Table 4) compared to those who did develop infection (19.8 mU/mg, $p = 0.02$). Twenty-four-hour leukocyte apoptosis levels in the two groups were similar (Fig. 2). These results suggest that leukocyte apoptosis is associated with protection against subsequent infection. The finding of increased leukocyte apoptosis pre-operatively also suggests the possibility of a genetic component to apoptosis of an individual's leukocytes in response to severe trauma.

To determine if increased leukocyte apoptosis is associated with protection from a particular type of infection or from more severe infection, we compared post-operative nucleosome levels within subgroups of T/HS patients who

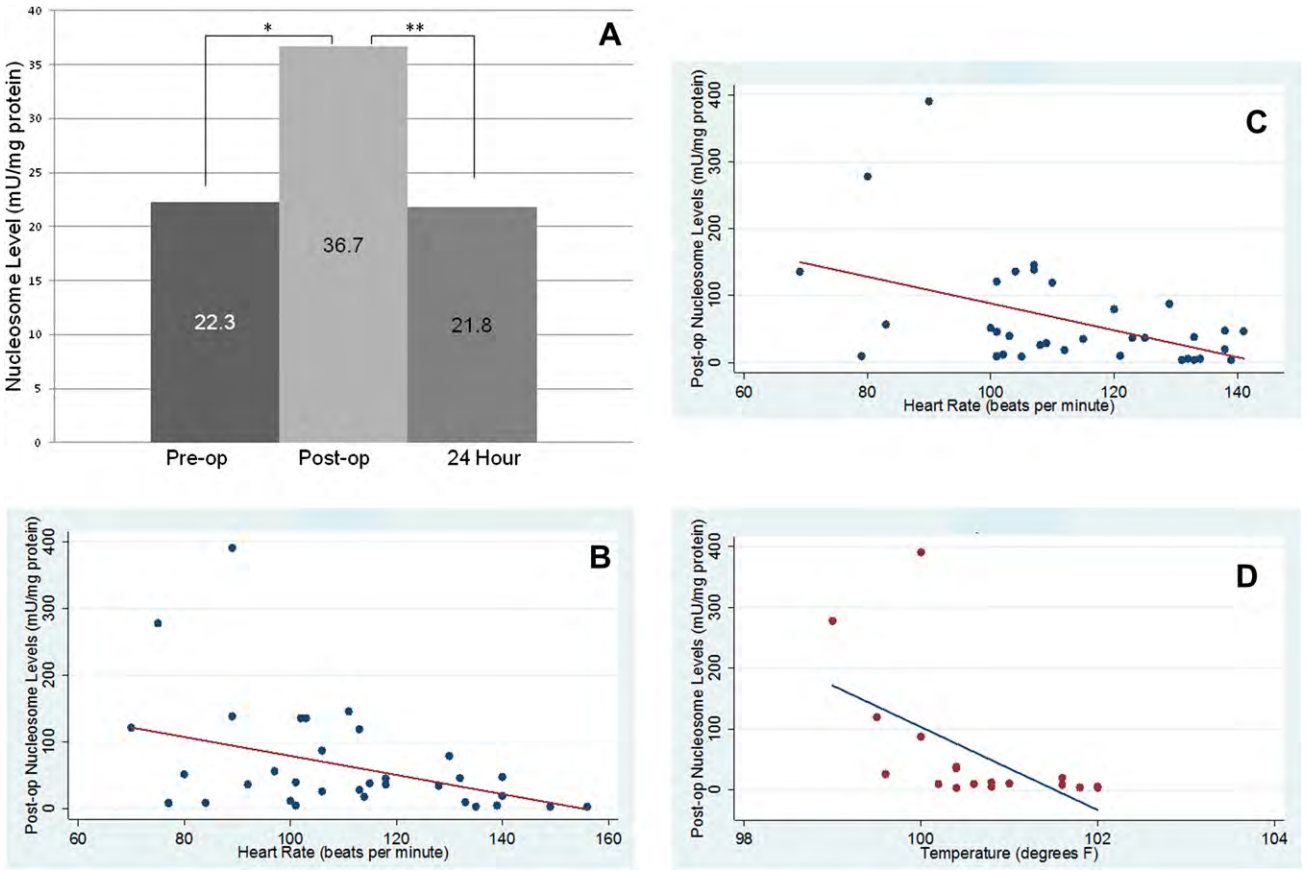



**Figure 1** Peripheral blood leukocyte apoptosis peaks immediately post-operatively (A) and is inversely correlated with heart rate at 24 and 48 h (B, C) and temperature at 48 h (D) during early resuscitation. In panel A, peripheral blood was collected from patients at three time points—pre-op, post-op and 24 hours—for isolation of leukocytes by dextran sedimentation. Cells were lysed using buffer provided in the Cell Death Detection ELISAplus® kit (Roche) and protein quantified using the Bradford method. Nucleosome ELISA was then performed as described by the manufacturer (Roche) and each value normalized to total protein content with lysate. Data plotted are the median value for each time point (actual value indicated within each bar). Statistical differences between groups were determined using the Wilcoxon signed rank test; *p*-values are indicated by asterisks: (*) 0.06 and (**) 0.03. In panels B and C, post-operative peripheral blood leukocyte nucleosome levels were plotted as a function of heart rate at 24 h (B) or 48 h (C) for each patient. Post-operative nucleosome levels were inversely related to heart rate at 24 h ($r = -0.36$, $p = 0.02$) and 48 h ($r = -0.39$, $p = 0.01$; linear regression modeling). In panel D, post-operative peripheral leukocyte nucleosome levels were plotted as a function of temperature at 48 h for each patient. Post-operative peripheral leukocyte nucleosome levels were inversely related to temperature at 48 h ($r = -0.38$, $p = 0.01$; linear regression modeling).

developed particular types of infections, had multiple infections, or had bacteremia/sepsis with those who did not develop these complications (Table 4). Post-operative nucleosome levels in patients who did not develop bacteremia/sepsis were 4.2 fold higher than in those who did develop bacteremia/sepsis ($p = 0.03$), while post-operative nucleosome levels in patients who did not develop multiple infections were 4.6-fold higher than in those patients who did develop multiple infections ($p = 0.01$). These findings suggest that leukocyte apoptosis post-operatively is associated with protection from bacteremia/sepsis and multiple infections, in particular. Similar results suggest that higher

**Table 3** Number of patients per infection category.

| | *n* | % of Total subjects ($N = 35$) |
|---|---|---|
| Pneumonia | 11 | 31 |
| Intra-abdominal abscess | 9 | 26 |
| Bacteremia and sepsis | 5 | 14 |
| Urinary tract infection | 4 | 11 |
| Wound infection | 4 | 11 |
| Other (empyema) | 1 | 3 |

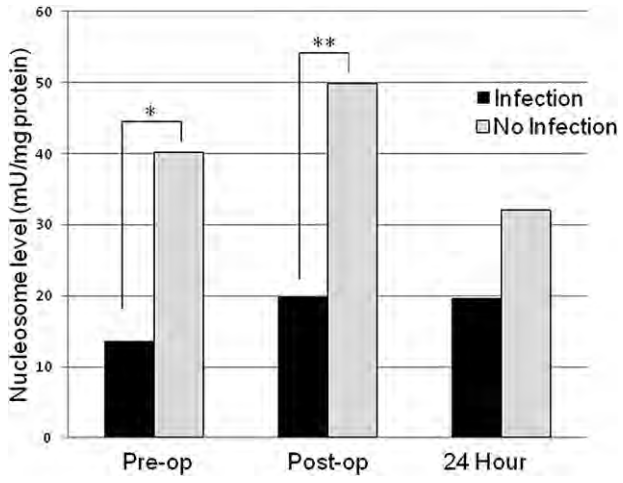



**Figure 2** Peripheral blood leukocyte nucleosome levels in patients with and without infection. Peripheral blood was collected from patients at three time points—pre-op, post-op and 24 hours—for isolation of leukocytes by dextran sedimentation. Cells were lysed using buffer provided in the Cell Death Detection ELISAplus® kit (Roche) and protein quantified using the Bradford method. Nucleosome ELISA was then performed as described by the manufacturer (Roche) and each value normalized to total protein content with lysate. Patients were followed for 30 days, or until death or hospital discharge for development of infection. Pre-operative, post-operative and 24 h median nucleosome levels of patients who developed infection were compared to patients who did not develop any infection. Statistical differences between groups were determined using the Wilcoxon signed rank test; *p*-values are indicated by asterisks: (*) 0.04, (**) 0.02, and (***) >0.05.

leukocyte apoptosis pre-operatively is associated with protection from bacteremia/sepsis (data not shown).

## Discussion

In this study, we demonstrated that peripheral leukocyte apoptosis occurs in T/HS patients, peaks immediately post-operatively, consists mostly of PMN, is inversely correlated with heart rate and fever at 24 and 48 h, and is higher pre-operatively and post-operatively in those destined to not develop infections, especially multiple infections and bacteremia plus sepsis. These results suggest that apoptosis of peripheral blood leukocytes, in particular PMN, early in hospitalized T/HS patients may protect patients from subsequent infection perhaps through reducing early PMN-mediated tissue injury.

Much of the disagreement in the literature over whether leukocyte apoptosis increases or decreases the risk of infection following hemorrhagic shock depends upon which leukocyte population is being examined. Studies involving lymphocytes have correlated increased levels of apoptosis within primary lymphoid organs such as the thymus and spleen with elevated risk of subsequent infection.[19–22] This is presumably due to the direct immunosuppressive effect of depleting functional lymphocytes within these organs. Regarding circulating blood lymphocytes, Teodorczyk-Injeyan et al suggested that injury related immune-deficiency correlated to increased T cell apoptosis.[23] Another recent study showed that high injury scores (SOFA) were linked to elevated circulating lymphocyte apoptosis markers (sFas, sFas/FAsL ratio) in patients with bacteremia.[24]

On the other hand, several studies of neutrophils in rodents have convincingly shown that increased levels of PMN apoptosis may actually reduce the severity of subsequent infection[25,26] and MOF.[10,27] Henrich et al suggest that trauma-activated PMNs release inflammatory mediators and contribute to tissue injury and delayed wound healing.[28]

Data from human studies are limited, and much remains to be learned. A recent study in trauma patients demonstrated an inverse correlation between degree of late neutrophil apoptosis and severity of end-organ dysfunction, however there was no significant difference between septic and non-septic patients.[29] The current study, to our knowledge, is the first to examine the impact of early peripheral blood PMN apoptosis on infectious outcomes in trauma patients with hemorrhagic shock. TUNEL staining of peripheral blood leukocyte cytospins in our current study demonstrated that 72% of the apoptotic leukocytes in the early post-operative period were PMNs. In keeping with these findings, and based upon other results presented in this paper, we propose that increased peripheral blood PMN apoptosis demonstrated early in the course of trauma-hemorrhagic shock may protect the severely injured trauma patient from developing subsequent infection, by one or both of two mechanisms. The first

**Table 4** Correlation between post-operative nucleosome levels in peripheral blood leukocytes and type of infection.

| | Infection | | No infection | | *p*-Value |
|---|---|---|---|---|---|
| | *n* | Median nucleosome level | *n* | Median nucleosome level | |
| Any infection | 17 | 19.8 | 18 | 49.8 | 0.02[a] |
| Pneumonia | 11 | 19.8 | 24 | 46.8 | 0.051 |
| Abdominal abscess | 9 | 26.1 | 26 | 39.0 | 0.57 |
| Sepsis | 5 | 10.2 | 30 | 42.8 | 0.03[a] |
| Urinary tract infection | 4 | 14.2 | 31 | 39.8 | 0.07 |
| Wound infection | 4 | 27.4 | 31 | 39.8 | 0.19 |
| Multiple infections | 9 | 10.2 | 26 | 46.8 | 0.01[a] |

[a] Analysis was performed using the Wilcoxon—Mann—Whitney test.

mechanism is through reducing immune-mediated tissue injury; the second mechanism involves restricted immunosuppression associated with clearance of apoptotic PMNs.

It is well established that PMNs play an integral role in the body's initial inflammatory response to trauma and are recruited to sites of injury.[6,10] Once sequestered, these cells possess tremendous potential to induce additional injury to the tissues to which they are recruited. Apoptotic PMNs within the circulation are removed by macrophages within the liver and spleen rather than infiltrating into organs and contributing to inflammation and further tissue injury. Preventing tissue injury and necrosis in this manner may reduce the risk that the organ will become a subsequent site of infection as well as the likelihood it will fail.

Tissue injury from infiltrating PMNs is not limited only to organs that have been directly injured from trauma. Ischemia-reperfusion injury (which occurs after successful resuscitation from hemorrhagic shock) also results in the recruitment of neutrophils to the reperfused organs. In fact, neutrophil-mediated organ injury following resuscitation from hemorrhage has been demonstrated in liver, heart, kidney, and intestine.[6] Additionally, activated PMNs stimulate macrophages to release inflammatory cytokines and chemotactic substances which further amplify the inflammatory response and increase the risk of developing MOF.[30] The presence of large numbers of activated PMNs has been repeatedly associated with pathogenesis of SIRS,[6,7,27] which itself is associated with increased risk of MOF and infection.[27,30] Patients with SIRS/MOF have been shown to have delayed PMN apoptosis,[7] enhanced PMN oxidative burst activity[7,10] and increased end-organ sequestration.[10] It therefore follows that if PMNs could be removed from the circulation before end-organ infiltration and therefore without inciting an inflammatory response, the risk of tissue injury and susceptibility to infection might be reduced.[26,27]

An alternative mechanism to explain our findings involves apoptosis-mediated immunosuppression. It is well established that apoptosis of PMN within tissue is accompanied by localized immunosuppression.[31,32] Ingestion of apoptotic cells by macrophages results in the release of anti-inflammatory mediators, including TGF-$\beta$1 and $PGE_2$[33] and suppresses the production of pro-inflammatory cytokines such as IL-8 and TNF-$\alpha$, as well as other pro-inflammatory mediators, including $TXA_2$.[32] Clearance of circulating apoptotic PMNs by the liver and spleen may restrict immunosuppression to these organs thereby sparing other organs such as the lung, which is among the most common sites of infection following serious trauma that leads to MOF and death.

It is clear from the above discussion that neutrophils are a critical component of the innate host defense and their apoptosis and removal are essential for efficient resolution of inflammation. What remains unclear, however, is how PMN apoptosis is regulated in humans. A recent study demonstrated delayed spontaneous, as well as microbe-related apoptosis, of neutrophils in 4 patients with auto-inflammatory disease, leukocytosis, and single-nucleotide polymorphisms (SNPs) in two genes encoding proteins that contribute to the formation of the caspase-1-activating NALP3 inflammasome complex, *NLRP3* (Q705K) and *CARD8* (C10X).[34] These findings implicate these two genes as part of the genetic program controlling PMN apoptosis in response to stress and infection. A notable finding in our study was that infected and uninfected patients segregated early in the pre-operative course and exhibited a significant difference in pre-operative leukocyte apoptosis following severe injury. This finding suggests that it might be desirable to intervene early in those individuals with low pre-operative neutrophil apoptosis in an effort to increase it. However, such an intervention would need to be selective for neutrophils and otherwise safe.

A limitation of this study is the relatively small sample size, which makes it difficult to determine significant differences between subgroups of infected patients. In several instances, differences just barely failed to reach statistical significance, and it is possible that with a larger sample size, these differences might become statistically significant. Almost half of the patients in the parent trial were excluded from this study because surgery was initiated before collection of a pre-operative blood sample. This could be regarded as a potential limitation of the study, even though how it may have biased the results is unclear. Another limitation is the presence of numerous potential confounders, which could not be controlled for. Also, multiple comparisons between the infected and non-infected groups were made for the sake of completeness and the differences observed in neutrophil apoptosis between the groups may be due to the increased chance of a difference between groups emerging in the setting of multiple comparisons. The significance of identifying 72% of apoptotic cells as PMNs may have been enhanced if complete blood counts and differentials were also performed. Clearly, PMNs predominated as the most common apoptotic cell in the peripheral blood, but in the absence of peripheral blood leukocyte counts differentials, it is not possible to determine the absolute number of apoptotic PMN and lymphocytes.

In conclusion, this study demonstrates that increased peripheral blood PMN apoptosis in the early post-operative period is associated with decreased risk of developing subsequent infection in severely injured trauma patients requiring emergency laparotomy or thoracotomy. These findings add additional support to the hypothesis that strategies aimed at limiting PMN number and function at an early point in the course of resuscitation period may be beneficial in this patient population.

## Acknowledgement

This work was supported, in part, by grant DM090899 from the Department of Defense to D.J.T.

None of the authors have any conflicts of interest to declare.

## References

1. Peden MMK, Sharma G. The injury chart book: a graphical overview of the global burden of injuries; 2002. Available from: http://whqlibdoc.who.int/publications/924156220x.pdf.
2. Heron M, Hoyert DL, Murphy SL, Xu J, Kochanek KD, Tejada-Vera B. Deaths: final data for 2006. *Natl Vital Stat Rep* 2009 Apr 17;**57**(14):1—134.

3. *United States, all injury deaths and rates per 100,000*. Office of Statistics and Programming, National Center for Injury Prevention and Control; 2007. CDC2007.
4. Baker CC, Oppenheimer L, Stephens B, Lewis FR, Trunkey DD. Epidemiology of trauma deaths. *American Journal of Surgery* 1980 Jul;**140**(1):144—50.
5. Sauaia A, Moore FA, Moore EE, Moser KS, Brennan R, Read RA, et al. Epidemiology of trauma deaths: a reassessment. *Journal of Trauma* 1995 Feb;**38**(2):185—93.
6. Moore FA, Moore EE. Evolving concepts in the pathogenesis of postinjury multiple organ failure. *Surgical Clinics of North America* 1995 Apr;**75**(2):257—77.
7. Jimenez MF, Watson RW, Parodo J, Evans D, Foster D, Steinberg M, et al. Dysregulated expression of neutrophil apoptosis in the systemic inflammatory response syndrome. *Archives of Surgery* 1997 Dec;**132**(12):1263—9. discussion 9—70.
8. Harter L, Mica L, Stocker R, Trentz O, Keel M. Mcl-1 correlates with reduced apoptosis in neutrophils from patients with sepsis. *The Journal of the American College of Surgeons* 2003 Dec;**197**(6):964—73.
9. Wesche DE, Lomas-Neira JL, Perl M, Chung CS, Ayala A. Leukocyte apoptosis and its significance in sepsis and shock. *Journal of Leukocyte Biology* 2005 Aug;**78**(2):325—37.
10. Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. *Journal of Trauma* 1995 Sep;**39**(3):411—7.
11. Cox G, Gauldie J, Jordana M. Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. *American Journal of Respiratory Cell and Molecular Biology* 1992 Nov;**7**(5):507—13.
12. Maianski NA, Roos D, Kuijpers TW. Bid truncation, bid/bax targeting to the mitochondria, and caspase activation associated with neutrophil apoptosis are inhibited by granulocyte colony-stimulating factor. *Journal of Immunology* 2004 Jun 1;**172**(11): 7024—30.
13. Maianski NA, Mul FP, van Buul JD, Roos D, Kuijpers TW. Granulocyte colony-stimulating factor inhibits the mitochondria-dependent activation of caspase-3 in neutrophils. *Blood* 2002 Jan 15;**99**(2):672—9.
14. Alten JA, Moran A, Tsimelzon AI, Mastrangelo MA, Hilsenbeck SG, Poli V, et al. Prevention of hypovolemic circulatory collapse by IL-6 activated Stat3. *PLoS ONE* 2008;**3**(2): e1605.
15. Moran A, Akcan Arikan A, Mastrangelo MA, Wu Y, Yu B, Poli V, et al. Prevention of trauma and hemorrhagic shock-mediated liver apoptosis by activation of stat3alpha. *International Journal of Clinical and Experimental Medicine* 2008;**1**(3):213—47.
16. Moran A, Tsimelzon AI, Mastrangelo MA, Wu Y, Yu B, Hilsenbeck SG, et al. Prevention of trauma/hemorrhagic shock-induced lung apoptosis by IL-6-mediated activation of Stat3. *Clinical and Translational Science*. 2009 Feb;**2**(1):41—9.
17. Morrison CA, Carrick MM, Norman MA, Scott BG, Welsh FJ, Tsai P, et al. Hypotensive resuscitation strategy reduces transfusion requirements and severe postoperative coagulopathy in trauma patients with hemorrhagic shock: preliminary results of a randomized controlled trial. *Journal of Trauma* 2011 Mar; **70**(3):652—63.
18. Tkatch LS, Rubin KA, Ziegler SF, Tweardy DJ. Modulation of human G-CSF receptor mRNA and protein in normal and leukemic myeloid cells by G-CSF and retinoic acid. *Journal of Leukocyte Biology* 1995 Jun;**57**(6):964—71.
19. Cobb JP, Buchman TG, Karl IE, Hotchkiss RS. Molecular biology of multiple organ dysfunction syndrome: injury, adaptation, and apoptosis. *Surgical Infections (Larchmt)* 2000;**Fall 1**(3): 207—13. discussion 14—5.
20. Hotchkiss RS, Swanson PE, Freeman BD, Tinsley KW, Cobb JP, Matuschak GM, et al. Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. *Critical Care Medicine* 1999 Jul;**27**(7):1230—51.
21. Laudanski K, Miller-Graziano C, Xiao W, Mindrinos MN, Richards DR, De A, et al. Cell-specific expression and pathway analyses reveal alterations in trauma-related human T cell and monocyte pathways. *Proceedings of National Academy of Sciences USA* 2006 Oct 17;**103**(42):15,564—15,569.
22. Delogu G, Moretti S, Antonucci A, Marcellini S, Masciangelo R, Famularo G, et al. Apoptosis and surgical trauma: dysregulated expression of death and survival factors on peripheral lymphocytes. *Archives of Surgery* 2000 Oct;**135**(10):1141—7.
23. Teodorczyk-Injeyan JA, Cembrzynska-Nowak M, Lalani S, Peters WJ, Mills GB. Immune deficiency following thermal trauma is associated with apoptotic cell death. *Journal of Clinical Immunology* 1995 Nov;**15**(6):318—28.
24. Huttunen R, Syrjanen J, Vuento R, Laine J, Hurme M, Aittoniemi J. Apoptosis markers soluble Fas (sFas), Fas Ligand (FasL) and sFas/FasL ratio in patients with bacteremia: a prospective cohort study. *Journal of Infection* 2012 Mar;**64**(3):276—81.
25. Kennedy AD, DeLeo FR. Neutrophil apoptosis and the resolution of infection. *Immunological Research* 2009;**43**(1—3):25—61.
26. Ren Y, Xie Y, Jiang G, Fan J, Yeung J, Li W, et al. Apoptotic cells protect mice against lipopolysaccharide-induced shock. *Journal of Immunology* 2008 Apr 1;**180**(7):4978—85.
27. Melley DD, Evans TW, Quinlan GJ. Redox regulation of neutrophil apoptosis and the systemic inflammatory response syndrome. *Clinical Sciences (London)* 2005 May;**108**(5):413—24.
28. Henrich D, Zimmer S, Seebach C, Frank J, Barker J, Marzi I. Trauma-activated polymorphonucleated leukocytes damage endothelial progenitor cells: probable role of CD11b/CD18-CD54 interaction and release of reactive oxygen species. *Shock* 2011 Sep;**36**(3):216—22.
29. Paunel-Gorgulu A, Kirichevska T, Logters T, Windolf J, Flohe S. Molecular mechanisms underlying delayed apoptosis in neutrophils from multiple trauma patients with and without sepsis. *Molecular Medicine* 2011 Dec 7.
30. Haslett C. Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. *Clinical Sciences (London)* 1992 Dec;**83**(6):639—48.
31. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. *Nature* 1997 Nov 27;**390**(6658):350—1.
32. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *Journal of Clinical Investigation* 1998 Feb 15; **101**(4):890—8.
33. Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *Journal of Clinical Investigation* 2002 Jan;**109**(1):41—50.
34. Blomgran R, Patcha Brodin V, Verma D, Bergstrom I, Soderkvist P, Sjowall C, et al. Common genetic variations in the NALP3 inflammasome are associated with delayed apoptosis of human neutrophils. *PLoS One* 2012;**7**(3):e31326.

Journal of Infection (2014) 68, 231–241

BIA
British Infection Association

www.elsevierhealth.com/journals/jinf

# Restoration of lung surfactant protein D by IL-6 protects against secondary pneumonia following hemorrhagic shock

Stephen Thacker [a], Ana Moran [b], Mihalis Lionakis [b], Mary-Ann A. Mastrangelo [b], Tripti Halder [b], Maria del Pilar Huby [b], Yong Wu [b], David J. Tweardy [b,c,d,*]

[a] *Section of Infectious Diseases, Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA*
[b] *Section of Infectious Diseases, Department of Medicine, Baylor College of Medicine, Houston, TX, USA*
[c] *Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA*
[d] *Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, USA*

Accepted 19 November 2013
Available online 27 November 2013

**KEYWORDS**
Unfolded protein response;
Hemorrhagic shock;
Pneumonia;
Alveolar epithelial cell

**Summary** *Objectives:* To identify novel approaches to improve innate immunity in the lung following trauma complicated by hemorrhagic shock (T/HS) for prevention of nosocomial pneumonia.
*Methods:* We developed a rat model of T/HS followed by *Pseudomonas aeruginosa* (PA) pneumonia to assess the effect of alveolar epithelial cell (AEC) apoptosis, and its prevention by IL-6, on lung surfactant protein (SP)-D protein levels, lung bacterial burden, and survival from PA pneumonia, as well as to determine whether AEC apoptosis is a consequence of the unfolded protein response (UPR). Lung UPR transcriptome analysis was performed on rats subjected to sham, T/HS, and T/HS plus IL-6 protocols. Group comparisons were performed via Kaplan–Meier or ANOVA.
*Results:* T/HS decreased lung SP-D by 1.8-fold ($p < 0.05$), increased PA bacterial burden 9-fold ($p < 0.05$), and increased PA pneumonia mortality by 80% ($p < 0.001$). IL-6, when provided at resuscitation, normalized SP-D levels ($p < 0.05$), decreased PA bacterial burden by 4.8-fold ($p < 0.05$), and prevented all mortality from PA pneumonia ($p < 0.001$). The UPR transcriptome was significantly impacted by T/HS; IL-6 treatment normalized the T/HS-induced UPR transcriptome changes ($p < 0.05$).

* Corresponding author. Section of Infectious Diseases, BCM 286, Room N1318, One Baylor Plaza, Houston, TX 77030, USA. Tel.: +1 713 798 8908; fax: +1 713 798 8948.
*E-mail address:* dtweardy@bcm.edu (D.J. Tweardy).


http://dx.doi.org/10.1016/j.jinf.2013.11.010

*Conclusions:* Impaired innate lung defense occurs following T/HS and is mediated, in part, by reduction in SP-D protein levels, which, along with AEC apoptosis, may be mediated by the UPR, and prevented by use of IL-6 as a resuscitation adjuvant.


## Introduction

Nosocomial pneumonia is the most common cause of death in patients suffering trauma complicated by hemorrhagic shock (T/HS) who survive their initial injuries.[1] While significant strides have been made in identifying the clinical findings and laboratory parameters associated with onset of pneumonia following traumatic injuries, the molecular basis for predisposition to pneumonia in T/HS is not fully understood. The concept of immune paralysis or immunodepression in patients following T/HS has growing support.[2,3] However, details of how immunodepression develops and its subsequent impact on the host have not been fully elucidated, particularly with regards to innate immunity, nor have specific measures emerged to prevent it.

A key component of innate immune defense in the lung is surfactant protein (SP)-D. SP-D is a member of the collectin family of proteins, which have a carboxy-terminal domain with calcium-dependent lectin activity. This lectin domain mediates the lectin:pathogen interaction, leading to pathogen aggregation, opsonization and enhanced pathogen phagocytosis, as well as a direct bactericidal effect.[4] SP-D has been shown to be critical in the innate host defense of the lung protecting against various inhaled pathogens and allergens.[5,6] Indeed, SP-D null mice have demonstrated increased susceptibility to multiple pathogens,[7] and SP-D has been shown to bind and aggregate *Pseudomonas aeruginosa*, one of the most commonly encountered pathogens in ventilator-associated pneumonia (VAP).[8–10]

SP-D, as with other surfactant proteins, is largely produced by type II alveolar epithelial cells (AECII).[11] AECII are found within the alveolar space, forming the extensive alveolar epithelial lining of the lung in conjunction with type I alveolar epithelial cells (AECI). AECII constantly produce surfactant proteins, such as SP-D, that are extruded into the extracellular space in an exocytic fashion to help maintain the surfactant layer, a key component of innate lung defense.

Using a rat model of T/HS,[12–15] we previously demonstrated that up to 15% of AECII undergo apoptosis in the acute post-resuscitative phase, and that AECII injury/apoptosis can be prevented when IL-6 is used as a resuscitative adjuvant through a Stat3-mediated mechanism.[14] In this report, we investigated the hypothesis that AECII injury/apoptosis contributes to pneumonia susceptibility in T/HS and that this contribution is mediated, in part, through reductions in SP-D levels. We found that T/HS decreased lung SP-D levels by almost half, which was associated with a 9-fold increase in lung bacterial burden and a 80% increase in mortality from PA pneumonia. IL-6, when provided at resuscitation to T/HS rats, normalized lung SP-D levels, decreased bacterial burden, and prevented all mortality from PA pneumonia. Analysis of the UPR transcriptome supports the hypothesis that the UPR contributes to AECII apoptosis following T/HS and its prevention by IL-6. These findings provide new opportunities for preventing nosocomial pneumonia in shock/trauma patients including use of IL-6 as a resuscitation adjuvant or administration of clinically available proteostasis modulators.

## Methods

### Rat T/HS protocol

These studies were approved by the Baylor College of Medicine Institutional Review Board for animal experimentation (Protocol AN-1980) and conform to National Institutes of Health guidelines for the care and use of laboratory animals. Adult male Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN). Rats were subjected to the sham or T/HS protocols, as described[12–14,16,17] with modifications. Under inhaled isoflurane anesthesia, both superficial femoral arteries (SFA) were cannulated. The right SFA site was used for continuous blood pressure monitoring and the left SFA site was used for blood withdrawal and fluid administration. Animals subjected to T/HS, underwent an initial bleed of 2.25 ml/100 g body weight over 10 min to achieve a target mean arterial blood pressure (MAP) of 35 mmHg, maintained for a period of ~3 h (mean duration = 191 ± 2.5 min) by episodically withdrawing or returning shed blood. Sham rats were anesthetized and cannulated for a total of ~4 h (a time period encompassing both the hypotensive and resuscitation phases), but were not subjected to hemorrhage or resuscitation. At the end of the hypotensive period, rats in the T/HS-PBS groups were resuscitated with our standard protocol (infusion of the remaining shed blood and two times the total shed blood volume with Ringer's lactate over 30 min starting with a 0.1 ml bolus of PBS) or they received IL-6 (10 μg/kg in 0.1 ml PBS via intra-arterial catheter) at the start of standard resuscitation (T/HS-IL6). We previously performed dose-response-studies, in which four doses of IL-6 (1, 3, 10 and 30 μg/kg) were tested, and demonstrated that 10 μg/kg was the optimum concentration to prevent organ injury (Tweardy et al., 2002, unpublished). To assess the effect of T/HS without or with IL-6 on the UPR transcriptome, 4 rats from each group were sacrificed 1 h after the start of resuscitation while under anesthesia and their lungs were harvested for RNA isolation and microarray analysis. To assess the effect of sham or T/HS without or with IL-6 on susceptibility to pneumonia, femoral wounds were closed surgically and anesthesia was reversed. Animals were given analgesia, returned to their cages, observed overnight and allowed to ambulate and feed *ad libitum* before subjecting them to the PA pneumonia protocol (see below). The survival rate in rats subjected to the T/HS protocol without or with IL-6 is ~100% at 24 h and beyond.

## Bacterial strain and inoculum preparation and quantification

*P. aeruginosa* (PA) strain ATCC-27853 (PA; a kind gift from Dr. John Alverdy, University of Chicago, IL) was used in all experiments. The target inoculum size of $3 \times 10^7$ CFU was determined as optimal in dose-survival experiments (0.03, 0.1, 0.3, and $1 \times 10^9$ CFU) in normal healthy Sprague—Dawley rats based on earlier studies in a rat intra-tracheal inoculum PA pneumonia model.[18,19] The mortalities observed with each inoculum were 17%, 20%, 75%, and 100%, respectively. The 17% mortality observed with $3 \times 10^7$ CFU was assessed as optimum based on our earlier results demonstrating increased susceptibility to intraperitoneal *Staphylococcus aureus* infection in mice following T/HS.[20] The target inoculum size ($3 \times 10^7$ CFU) was obtained by a broth culture prepared by isolating a single colony from an agar plate grown at 37 °C for 15—17 h in trypticase-soy agar (TSA; Becton, Dickinson and Company. Sparks, MD, USA) and inoculating it into trypticase-soy broth (TSB; Becton, Dickinson and Company. Sparks, MD, USA). The broth was incubated at 37 °C and the optical density (OD) measured to achieve the OD corresponding to the target inoculum size, which was then confirmed by serial dilution and culture on TSA plates.

## T/HS-pneumonia protocols

Twenty-four hours after being subjected to the sham or T/HS protocol without or with IL-6, rats were given a sublethal dose of PA (mean inoculum size $3.1 \pm 0.2 \times 10^7$ CFU) through the transtracheal route. Briefly, a 1 cm incision in the anterior aspect of the neck was done under 2% isoflurane anesthesia, the fascia and muscle layers were dissected and the trachea exposed. The bacterial inoculum in a volume of 0.2 ml of PBS was transtracheally instilled through a 22-gauge needle inserted into the trachea, followed by 0.5 of air for uniform inoculum distribution. The incision was surgically closed. Rats were administered analgesia, allowed to recover in their cages, and observed every 6 h for 48 h to quantify survival (survival protocol; Fig. 1) or sacrificed 4 h after intratracheal inoculation and lungs harvested for lung bacterial burden quantification (bacterial burden protocol, Fig. 2). After sacrifice, lungs from rats subjected to the bacterial burden protocol were collected, weighed, and homogenized in 2 ml PBS. Serial log dilutions of organ homogenate (1:10, 1:100, and 1:1000) were made and plated on TSA plates in duplicate. Plates were incubated at 37 °C overnight after which bacterial CFU were counted. Results are presented as CFU/gm tissue weight.

## Lung protein extraction and protein quantitation

Frozen lungs of rats subjected to sham or T/HS protocol without or with IL-6 and harvested at 1 h after end of resuscitation were cut by cryotome, resuspended in high salt buffer, and sonicated in ice 3 times, 10 s each, as previously described.[12—15] Samples were then centrifuged 15 min at 5000 RPM and the supernatant collected and evaluated by Bradford assay for total protein quantification.

## Myeloperoxidase (MPO) staining

Paraformaldehyde-fixed and paraffin-embedded lung sections were rehydrated from Xylene to PBS through a series of decreasing concentrations of ethanol and placed in a DAKO autostainer. MPO rabbit polyclonal antibody (Lab Vision, Corp.) was used at the provided concentration. The horseradish peroxidase (HRP) system for rabbit antibodies was used as per the manufacturer's instructions. Slides were counterstained with hematoxylin. MPO-positive cells were assessed microscopically in 20 random 1000× high power fields (hpf) by an experienced histologist. Data is presented as the number of MPO-positive cells/hpf.

## Immunoblotting

Levels of surfactant protein (SP)-D in high-salt protein extracts of frozen lungs were assessed by immunoblotting with mouse monoclonal antibody to SP-D (Santa Cruz Biotechnology, Santa Cruz CA). Protein samples—total lung protein (50 μg), recombinant rat SP-D (2.5 μg) and protein standards (SeeBlue® Plus2 Pre-stained Standards, Invitrogen; 7 μl) were separated by Tris-Glycine SDS-PAGE and transferred to a PVDF membrane. Recombinant rat SP-D was purified from CHO K1 cells and was variably glycosylated[21]; it was the kind gift of Dr. Erika Crouch, Washington University, St. Louis, MO. The membrane was incubated overnight with mouse monoclonal antibody and subsequently incubated with goat anti-mouse antibody conjugated with horseradish peroxidase (HRP; Zymed, San Francisco, CA) for 1 h. ECL agent (Amersham Biosciences, UK) was used for detection. Densitometry was performed using ImageJ 1.4g software (National Institutes of Health, Bethesda, MD).

## Microarray analysis

Gene expression profiling was performed with the Affymetrix Rat Array RAE 230A genechip following Affymetrix protocols used within the Baylor College of Medicine Microarray Core Facility. Genechips were hybridized with RNA isolated from the lungs of rats within each of three groups—sham, T/HS-PBS and T/HS-IL6—one chip for each of four lung RNA samples per group. Total RNA was isolated from 4 to 5 micron cryotome sections of each lung using TRIzol® Reagent (Invitrogen, Carlsbad, California) single step RNA isolation protocol followed by purification with RNeasy® Mini Kit (QIAGEN, Hilden, Germany) as instructed by the manufacturer. We used GenespringGX (Agilent Technologies Inc, Santa Clara CA) software package for quality assessment, statistical analysis and annotation. Low-level analyses included background correction, quartile normalization and expression estimation using RMA-based analysis within Genespring. One-way analysis of variance (ANOVA) with contrasts was used for group comparisons on all genes and on the list of UPR entities. *p*-Values were adjusted for multiple comparisons using the Benjamini-Hockberg method. The adjusted *p*-values represent false discovery rates (FDR) and are estimates of the proportion of "significant" genes that are false or spurious "discoveries". We used a False Discovery Rate (FDR) of 5% as cut-off. RAE

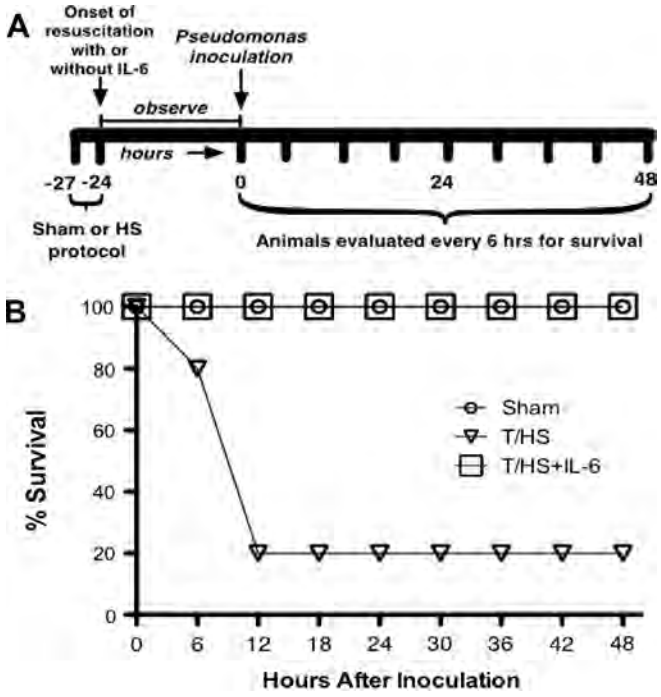



**Figure 1** Effect of T-HS on mortality due to PA pneumonia. Panel A depicts the sequence of interventions in the rat pneumonia survival protocol. Rats ($n = 10$ per group) were subjected to either the sham [triangle], T/HS [square], or T/HS + IL-6 resuscitation [circle] protocol over 3 h followed 24 h later by transtracheal inoculation of PA, then observed 48 h for survival. Survival (panel B) of T-HS rats was reduced 80% compared to that of sham rats ($p < 0.001$, Kaplan–Meier analysis); reduction in survival by T/HS was reversed by administration of IL-6 ($p < 0.001$, Kaplan–Meier analysis).

230A genechips each contained 15,923 probe sets representing 13,521 annotated genes or expressed sequence tags. A UPR gene entity list was created using both Ingenuity Pathway Analysis (IPA® Redwood City, CA) and the Gene Ontology Database©, with keywords "endoplasmic reticulum stress, unfolded protein response".

### Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Multiple group comparisons of means were done by one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test. Survival analysis was done by Kaplan–Meier test.

## Results

### T/HS increased mortality and bacterial burden in a model of *P. aeruginosa* pneumonia

To determine the effect of T/HS on susceptibility to PA pneumonia, we developed a pre-clinical model of T/HS combined with PA pneumonia in rats (Fig. 1A). Rats were subjected to the sham or T/HS protocol followed 24-h later by transtracheal inoculation of PA. After inoculation, rats were observed for survival for 72 h. Remarkably, survival in T/HS rats was decreased by 80% compared to sham rat group ($p < 0.001$, Kaplan–Meier analysis; Fig. 1B).

To determine if the cause of increased mortality observed in T/HS rats is due to increased bacterial burden in the lungs of these animals, we measured bacterial numbers in the homogenates of lungs of the rats subjected to sham or T/HS protocols 4 h after transtracheal inoculation of PA (Fig. 2). The PA CFU/gram of lung tissue was 9-fold higher in the rats subjected to T/HS protocol compared to sham rats ($p < 0.05$, ANOVA) strongly suggesting that the increased mortality of T/HS mice following PA inoculation was due to increased bacterial burden.

To assess if the increased bacterial burden in T/HS rats is due to a decrease in infiltrating neutrophils resulting in impaired clearance of bacteria, we performed myeloperoxidase staining of lung sections 4 h after inoculation with PA. The number of MPO-positive cells in the lungs of PA-infected T/HS rats (35 ± 5 cells per 1000× field; mean ± SEM) was identical to the number of MPO-positive cells in the lungs of PA-infected sham rats (34 ± 4 cells per 1000× field; mean ± SEM). In addition to there being no difference between groups in the number of MPO-positive cell within the lung, the distribution of MPO-positive cells within the alveoli and lung interstitium was similar (data not shown). Thus, the increased bacterial burden in T/HS rats was not due to differences in the quantity or distribution of PMN recruitment into the lung.

We previously demonstrated that AECII were the predominant cell type that underwent apoptosis following T/HS.[14] Since AECII produce SP-D, which increases PMN-mediated phagocytosis of bacteria including PA, we assessed SP-D levels in the lungs of T/HS and sham rats (Fig. 3). SP-D levels in the lungs of rats subjected to T/HS were reduced by 50% compared to lungs of sham rats at 24 h ($p < 0.05$, ANOVA). Thus, our data suggest that the

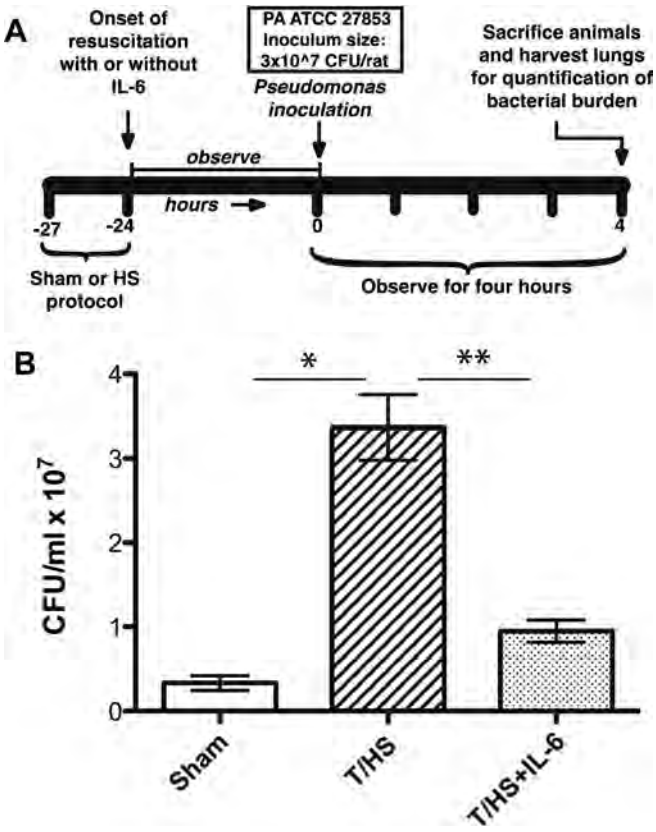



**Figure 2** Effect of T-HS on lung bacterial burden in the PA pneumonia. Panel A depicts the sequence of interventions in the rat pneumonia bacterial burden protocol. Rats ($n = 6$ per group) were subjected to either the sham or T/HS protocol over 3 h near the end of which they either received or did not receive IL-6. Twenty-four hr later, rats received a transtracheal inoculation of PA followed by sacrifice 4 h later. Bacterial CFU were counted in lung homogenates. Data are presented as bacteria CFU/gram of lung tissue (mean ± SEM). Bars with paired single or double asterisks above (*, **) differ significantly ($p < 0.05$, ANOVA).

increase in bacterial burden in the lungs of T/HS rats is due to reduced levels of SP-D in the lungs.

## Administration of IL-6 as a resuscitation adjuvant in T/H rats prevented *P. aeruginosa* pneumonia mortality, reduced bacterial lung burden, and normalized SP-D levels in the lung

We previously demonstrated that IL-6 administration as a resuscitation adjuvant induced activation of Stat3, particularly Stat3$\alpha$, within lung parenchymal cells resulting in protection against alveolar epithelial cell apoptosis following T/HS.[14] To assess if this prevention of apoptosis by IL-6 also affected nosocomial pneumonia mortality, rats were subjected to our T/HS protocol with IL-6 administered as a resuscitation adjuvant, followed 24 h later by transtracheal inoculation of PA (Fig. 1B). Impressively, animals that received IL-6 were completely protected against mortality ($p < 0.001$, Kaplan—Meier analysis). When we assessed how IL-6 affected bacterial burden (Fig. 2B), we found that lung bacterial burden was decreased by 4.8-fold in T/HS-IL6 rats compared to lung bacterial burden in T/HS-PBS rats ($p < 0.05$, ANOVA) to levels statistically indistinguishable from sham rats inoculated with PA.

To determine if lung bacterial burden was reduced in IL-6-treated T/HS rats as a result of restoration of SP-D levels, we assessed the effect of IL-6 treatment on SP-D levels in the lung. Densitometry analysis of immunoblots of whole lung homogenates from IL-6-treated T/HS rats (Fig. 3) revealed that IL-6 treatment prevented the reduction in pulmonary SP-D seen in T/HS animals ($p < 0.05$, ANOVA) with SP-D protein levels in the lungs of IL-6-treated T/HS rats being equivalent to sham animals.

## The UPR is significantly altered in the lung following T-HS and demonstrated normalization when IL-6 is used as a resuscitation adjuvant

The molecular mechanisms underlying T/HS-induced AECII apoptosis are not understood. We previously investigated the potential contribution of the classical intrinsic and extrinsic pathways to lung apoptosis in T/HS and demonstrated that T/HS altered the expression of many of intrinsic and extrinsic apoptosis pathway-related genes and that IL-6 treatment normalized expression of the majority of those genes altered by T/HS through a Stat3-dependent mechanism.[14] However, which genes were critical for apoptosis of AECII and its prevention, if any, were not specifically delineated in these studies.

The unfolded protein response (UPR) is a critical homeostatic mechanism for highly secretory cells such as AECII. Recently, the UPR has been described as a major

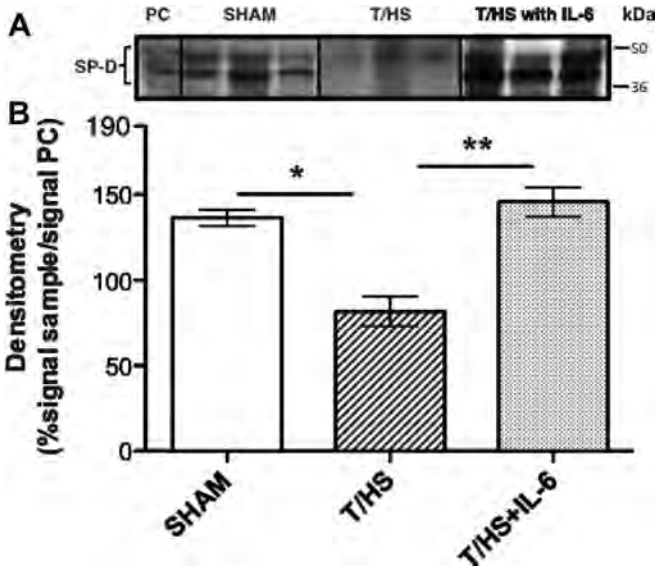



**Figure 3** Effect of T-HS on lung surfactant protein-D (SP-D) levels. Rat recombinant SP-D (2.5 μg; positive control, PC) or whole lung protein extracts (50 μg) from rats ($n = 3$ per group) subjected to sham or T/HS protocol were separated by SDS-PAGE and immunoblotted with monoclonal antibodies to SP-D (panel A). The band signal intensity was quantitated by densitometry and reported as a ratio of signal intensity in sample to signal intensity in positive control (PC) times 100. Bars with paired single or double asterisks above (*, **) differ significantly ($p < 0.05$, ANOVA).

cause of apoptosis when secretory cells are exposed to overwhelming or prolonged endoplasmic reticulum (ER) stress as would occur in T/HS. Since a central feature of the UPR is activation of several key transcription factors such as DDIT3 (CHOP), ATF4, ATF6, and XBP1, we investigated the impact of T/HS on the ER stress response at the transcriptome level by mining oligonucleotide microarray (Affymetrix) data previously obtained and archived by us from the lungs of 3 groups of rats: Sham, T/HS-PBS, and T/HS-IL6, sacrificed 1 h after the end of resuscitation.[14] A broad 185-gene UPR-associated entity list was generated following an extensive literature review and with the help of Ingenuity Pathway Analysis (IPA®). Of the 185-gene UPR set, 113 distinct gene entities were annotated and expressed across the chips after spot duplicates were removed. Importantly, the three experimental groups self organized based on this UPR-associated gene entity list expression (Fig. 4).

In order to determine the effect of the UPR on the observed T/HS-induced lung apoptosis and its prevention with IL-6, we performed intergroup comparisons of the transcriptome profiles within these groups. Within the list of 113 genes expressed by all groups, 65 (57%) were significantly impacted by T/HS when compared to Sham animals (ANOVA, $p < 0.05$). When we assessed for IL-6 responsive transcripts that proved significantly impacted by T/HS, we found that 53 (47%) entities were significantly altered in both T/HS vs. Sham and T/HS-IL6 vs. T/HS comparator groups. Thirty-two of these UPR-associated genes demonstrated $\geq$1.5-fold change in T/HS vs. Sham, 14 of which are considered pro-apoptotic (Table 1). Taking the known apoptotic function of these genes into context, we demonstrated that 86% (12 of 14) of the UPR-associated genes with known pro-apoptotic function were up-regulated following T/HS and subsequently normalized with IL-6. Among the most up-regulated genes within this intergroup comparison were pro-apoptotic UPR members, eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2; increased 4.7 fold by T/HS), DNA-damage-inducible transcript 3 (DDIT3; increased 2.5 fold by T/HS; also known as C/EBP-homologous protein, CHOP). Canonical UPR members, X-box binding protein 1 (XBP1) and activating transcription factor 4 (ATF4) also were both increased by 1.5 fold by T/HS when compared to sham (Table 1). In animals in which pulmonary cell apoptosis was prevented by receiving IL-6 at resuscitation, we find that these potentially pro-apoptotic UPR member transcripts (EIF2AK2, DDIT3, XBP1, and ATF4) are reduced to levels statistically indistinguishable from sham levels, strongly suggesting a contribution to prevention of pulmonary cell apoptosis (Table 1).

## Discussion

To investigate the impact of T/HS on the innate host defense of the lung, we developed a rodent model of secondary *P. aeruginosa* pneumonia following T/HS. We demonstrated in this model that T/HS increased lung bacterial burden 9-fold and resulted in an 80% increase in mortality. Neutrophil recruitment to the lung was not altered in infected T/HS lungs compared to infected sham lung to explain impaired bacterial clearance; rather, lung SP-D protein levels were decreased by nearly 50%. Use of IL-6 as a resuscitation adjuvant prevented the decrease in lung SP-D, reduced lung PA bacterial burden nearly 5-fold and completely prevented PA-mediated mortality. The UPR transcriptome of the lung was found to be significantly impacted by T/HS and to be normalized when IL-6 is given as a resuscitative adjuvant. These findings indicate that

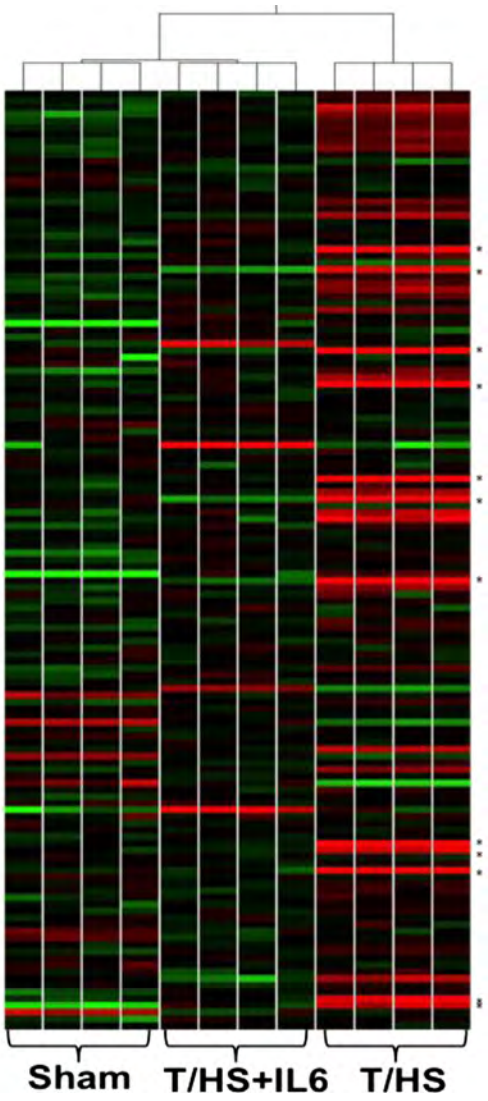



**Figure 4** Self-organizing heat map of UPR transcriptomes. Self-organizing heat map of UPR-associated gene entity list mRNA levels demonstrates clustering of experimental groups ($n = 4$ per group) and relative relatedness based on their expression profiles. Levels of relative expression range from bright green (−2-fold) to bright red (+2-fold). Genes marked with an asterisk (*) represent the first twelve pro-apoptotic genes listed in Table 1.

there is a maladaptive reduction in innate lung defense in T/HS mediated by a reduction in SP-D, which accompanies AECII apoptosis and contributes to increased susceptibility to pneumonia. AECII apoptosis may be mediated by the UPR, which, along with increased susceptibility to pneumonia, may be prevented by use of IL-6 as a resuscitative adjuvant.

AECII are referred to by some authors[22,23] as the "Defender of the Alveolus". They contribute to the mechanical viability of the alveolus by producing the pulmonary surfactant layer and are responsible for restoring injured alveolar epithelium. In addition, they have a unique role in the innate immunity of the lung. AECII have been shown to contribute to host defense by secreting anti-inflammatory and anti-microbial proteins into the alveolar space.[22,24] Most notable of these anti-microbial proteins are the collectins, SP-A and SP-D. SP-D, in particular, is known to enhance phagocytosis of *Pseudomonas*.[25,26] Given our previous findings that AECII was the pulmonary cell type undergoing the majority of the apoptosis caused by T/HS,[14] we hypothesized that loss of these cells may contribute to impaired innate host defense of the lung following T/HS. We began to address the role of the AECII in innate host defense by determining levels of SP-D in the lung of animals in our model of T/HS. SP-D is a good marker of AECII function as these cells are the predominant source of SP-D production. SP-D was reduced by nearly 50% in the whole lung of animals undergoing T/HS when compared to sham, which provides a unifying explanation for our observed findings that a relative deficiency of SP-D in the lung allows for increased PA growth leading to increased bacterial burden and increased pneumonia mortality.

Supporting the hypothesis that decreased SP-D predisposes to pneumonia is a recent study demonstrating that children with absent SP-D more frequently have pneumonia[27] and a recent study of acute lung injury caused by intestinal ischemia/reperfusion injury, which demonstrated a significant reduction of SP-D in the lung by immunohistochemistry staining.[28] Similarly, other investigators have shown that early in the evolution of acute lung injury, alveolar epithelial cell death leads to decreased production and increased clearance of SP-D.[29,30]

We have previously demonstrated the ability of IL-6-stimulated Stat3 to prevent AECII apoptosis following T/HS.[14] The exact mechanism of this protection is not fully understood despite prior investigation into the intrinsic and extrinsic cell death pathways.[14] Given the critical role of the UPR in highly secretory cell types such as AECII and its ability to drive these types of cells into apoptosis, we investigated the impact of T/HS with and without IL-6 on the UPR. Our investigation into the UPR suggests that this pathway contributes to the concert of stimuli that leads to cell death within the lung following T/HS. Previous work has demonstrated that the UPR is activated by trauma with hemorrhage demonstrating increased expression of ATF6, PERK, IRE$\alpha$, and CHOP and is associated with increased apoptosis within the liver.[31] Utilizing whole organ transcriptomic analysis, we examined the impact of T/HS on the UPR in the lung one hour following resuscitation, when apoptosis is maximal.[14] We identified several canonical members of the UPR with pro-apoptotic functions that demonstrate changes in transcript levels in response to T/HS and IL-6 intervention, which suggest this pathway may contribute to pro-apoptotic signaling. The most significantly impacted UPR members across all experimental comparisons were Eif2ak2, ATF4, CHOP (DDIT3), and XBP-1. ATF4 and XBP-1 have both been shown to be transcriptional activators of CHOP, which has been shown to be a potent stimulator of apoptosis through its downstream targets in many models.[32–35] Of note, previous work has shown that CHOP signaling mediates LPS-induced lung injury in a mouse model of sepsis, and when over-expressed in lung cell lines leads to increased apoptosis,[32] suggesting a role for CHOP in apoptosis of lung epithelial cells in settings of stress including T/HS.

**Table 1** Fold change comparisons (T/HS vs. Sham, T/HS + IL6 vs. T/HS, and T/HS + IL6 vs. Sham) for mRNA transcript levels with an absolute fold change of 1. 5-fold or greater T/HS vs. Sham comparison ($p < 0.05$, ANOVA).

| Gene symbol | Apoptotic role | Fold change | | |
|---|---|---|---|---|
| | | T/HS vs. Sham | T/HS + IL6 vs. T/HS | T/HS + IL6 vs. Sham |
| Eif2ak2 | pro | 4.7 | −4.2 | 1.1 |
| Tnf | pro | 2.8 | −1.7 | 1.6* |
| Ppp1r15a | pro | 2.8 | −1.3 | 2.1* |
| Ddit3 | pro | 2.5 | −3.6 | −1.4 |
| Casp12 | pro | 2.4 | −2.3 | 1.0 |
| Bid | pro | 2.1 | −1.9 | 1.1 |
| Casp3 | pro | 1.6 | −2.1 | −1.3 |
| Casp7 | pro | 1.6 | −1.6 | 1.0 |
| Atf4 | pro | 1.5 | −1.4 | 1.1 |
| Eif2s1 | pro | 1.5 | −1.2 | 1.3 |
| Tp53 | pro | 1.5 | −1.4 | 1.1 |
| Xbp1 | pro | 1.5 | −1.2 | 1.3 |
| Ccnd1 | pro | −1.8 | 1.2 | −1.5* |
| Pik3ip1 | pro | −2.3 | 1.7 | −1.4 |
| Apobec1 | anti | 3.2 | −3.9 | −1.2 |
| Psmb10 | anti | 2.6 | −2.7 | 1.0 |
| Psmb9 | anti | 2.5 | −3.3 | −1.3 |
| Psme2 | anti | 2.3 | −2.3 | 1.0 |
| Psmb2 | anti | 1.9 | −1.4 | 1.4 |
| Psmb8 | anti | 1.9 | −2.7 | −1.4 |
| Psma3 | anti | 1.8 | −1.3 | 1.4 |
| Psma1 | anti | 1.8 | −1.6 | 1.1 |
| Aars | anti | 1.8 | −1.7 | 1.1 |
| Psma5 | anti | 1.7 | −1.5 | 1.1 |
| Psma7 | anti | 1.7 | −1.5 | 1.1 |
| Psme1 | anti | 1.6 | −1.9 | −1.2 |
| Psmb3 | anti | 1.6 | −1.4 | 1.1 |
| Psma6 | anti | 1.5 | −1.1 | 1.4 |
| Vcp | anti | 1.5 | −1.3 | 1.2 |
| Hspb7 | anti | 1.5 | 2.0 | 3.0* |
| Hspalb | anti | −1.5 | 2.2 | 1.5* |
| Tor1b | unknown | 2.2 | −2.3 | 1.0 |

*$p < 0.05$; all other T/HS + IL6 vs. Sham comparisons $p > 0.05$.

In addition to 12 of the 14 pro-apoptotic gene transcripts that were increased with T/HS vs. Sham and normalized with IL-6 (Table 1), a pattern consistent with their contributing to lung apoptosis in T/HS and the protective effect of IL-6, there were several anti-apoptotic gene transcripts that were increased with T/HS vs. Sham and also were normalized with IL-6 (Table 1). These changes likely are a component of the lung's efforts to maintain homeostasis; T/HS-induced lung apoptosis and its prevention by IL-6 were accomplished in spite of their modulation. However, one UPR-related anti-apoptosis gene, Hspb7, a member of the small heat shock protein family (Table 1), was increased by T/HS vs. Sham and was further increased in the T/HS + IL-6 group while another UPR-related anti-apoptosis gene, Hspa1b, a member of the heat shock protein 70 family (Table 1), was decreased by T/HS vs. Sham and was increased in the T/HS + IL-6 group. Modulations in these two anti-apoptosis genes, perhaps like the 12 pro-apoptotic genes discussed above, also may have contributed to T/HS-induced AECII apoptosis and its prevention by IL-6.

Our results also indicate that IL-6 provides protection against PA pneumonia following T/HS. This protection is due, at least in part, to the ability of IL-6-activated Stat3 to protect AECII against T/HS-induced apoptosis as we previously demonstrated.[14] In this paper, we demonstrate that sparing the AECII from apoptosis maintains SP-D levels in the lung, which likely contributes to the protection against PA bacterial burden and mortality observed in our model of post-T/HS PA pneumonia.

Nosocomial pneumonia including ventilator-associated pneumonia (VAP) is one of the leading causes of healthcare-associated infection following severe trauma[1] and the most common cause of death in patients surviving the original traumatic injury. VAP following severe trauma is most often due to PA[8,36,37] with infection with antibiotic-resistant PA[38,39] and other Gram-negative bacteria[40] increasing in incidence at an alarming rate. Our

model allows for investigation not only of the mechanisms of pathogenesis of pneumonia following T/HS, but also allows us to begin to investigate novel interventions that may prevent PA pneumonia following T/HS. One category of intervention involves agents that might prevent AECII apoptosis and the resultant SP-D deficiency, while another category of intervention involves restoration of impaired innate epithelial cell immunity within the lung. Within the first category is the use of IL-6 as a resuscitation adjuvant. We have established that IL-6 as a resuscitation adjuvant is of clear benefit in preventing organ apoptosis and inflammation in rat and porcine models of T/HS,[12–15,41] as well as in reducing the severity of illness in pre-clinical models of bloodstream infections.[20] In addition to potentially preventing AECII apoptosis and pneumonia susceptibility in T/HS patients, this intervention also may prevent heart and liver dysfunction in T/HS by preventing apoptosis of cardiomyocytes[12] and hepatocytes.[13] However, the FDA has not approved IL-6 for this or any other indication. An alternative intervention that may be able to prevent AECII apoptosis and subsequent decrease in SP-D is use of proteostasis modulators such as geranylgeranylacetone (GGA; teprenone; Selbex®). GGA is an antiulcer drug that has been used in Japan for over thirty years and has a favorable side effect profile. GGA induces expression of HSP70 and HSP90; HSP90, in particular, has been shown to downregulate apoptosis secondary to UPR.[42,43] In fact, when given to rats during intracerebral hemorrhage, GGA decreased neuronal cell apoptosis and improved neurological recovery by increasing Stat3 activity.[44] In studies underway, we are examining the potential benefit of GGA in our rat model of T/HS to determine if it can be used to prevent apoptosis of AECII, reduction in SP-D, and susceptibility to PA pneumonia.

Two potential interventions that may restore lung innate immunity following T/HS-induced AECII apoptosis also are suggested by our findings—one is aerosol administration of SP-D; the second is inhaled Pam2-ODN, a combination of Toll-like receptor (TLR) agonists. Surfactant therapy has been used extensively and successfully in reducing mortality from respiratory distress syndrome of the newborn.[45] However, a significant proportion of infants born at less than 28 weeks' gestation develop neonatal chronic lung disease. Current surfactant therapies lack SP-D, yet animal models support a role for SP-D in reducing inflammation and infection in the lung, which suggests that supplementation of current surfactant therapies with recombinant forms of SP-D may help offset the risk of development of chronic lung disease. Thus, surfactant preparations containing SP-D may be available in the future to test in T/HS patients for the ability to reduce nosocomial pneumonia. Pam2-ODN consists of Pam2CSK4, a diacylated lipopeptide ligand for TLR2/6, combined with oligonucleotide (ODN) M362, a ligand for TLR9.[46] Pam2-ODN has been demonstrated to broadly protect mice against otherwise lethal pneumonias including those caused by *P. aeruginosa* and *Streptococcus pneumonia*.[46] Thus, an alternative to SP-D is Pam2-ODN inhalation to therapeutically boost residual lung epithelial cell intrinsic defenses following T/HS and potentially to protect T/HS patients from VAP.

## Acknowledgments

Supported, in part, by grants HL07619 (DT), HL66991 (DT), and AI055413 (ST, AM) from the National Institutes of Health, grant W81XWH-11-2-0018 (DT) from the US Army and H48839 (AM) from the American Lung Association. We would like to acknowledge Dr. John Alverdy (University of Chicago, Chicago, IL) for kindly providing the PA ATCC-27853 strain used in our experiments and Dr. Erica C. Crouch (Washington University, St. Louis, MO) for rat recombinant SP-D used in our experiments.

## References

1. Sauaia A, Moore FA, Moore EE, Moser KS, Brennan R, Read RA, et al. Epidemiology of trauma deaths: a reassessment. *The Journal of Trauma* 1995 Feb;**38**(2):185–93.
2. Angele MK, Faist E. Clinical review: immunodepression in the surgical patient and increased susceptibility to infection. *Critical Care (London, England)* 2002 Aug;**6**(4):298–305 [Review].
3. Kimura F, Shimizu H, Yoshidome H, Ohtsuka M, Miyazaki M. Immunosuppression following surgical and traumatic injury. *Surgery Today* 2010 Sep;**40**(9):793–808 [Review].
4. Wu H, Kuzmenko A, Wan S, Schaffer L, Weiss A, Fisher JH, et al. Surfactant proteins A and D inhibit the growth of gram-negative bacteria by increasing membrane permeability. *The Journal of Clinical Investigation* 2003 Jun;**111**(10):1589–602.
5. Crouch E, Wright JR. Surfactant proteins a and d and pulmonary host defense. *Annual Review of Physiology* 2001;**63**: 521–54 [Review].
6. Wright JR. Immunoregulatory functions of surfactant proteins. *Nature Reviews Immunology* 2005;**5**(1):58–68 [Review].
7. Shepherd VL. Distinct roles for lung collectins in pulmonary host defense. *American Journal of Respiratory Cell and Molecular Biology* 2002 Apr 01;**26**(3):257–60 [Review].
8. Babcock HM, Zack JE, Garrison T, Trovillion E, Kollef MH, Fraser VJ. Ventilator-associated pneumonia in a multihospital system: differences in microbiology by location. *Infection Control and Hospital Epidemiology: the Official Journal of the Society of Hospital Epidemiologists of America* 2003 Nov 01;**24**(11):853–8 [Comparative Study].
9. Douda DN, Jackson R, Grasemann H, Palaniyar N. Innate immune collectin surfactant protein D simultaneously binds both neutrophil extracellular traps and carbohydrate ligands and promotes bacterial trapping. *Journal of Immunology (Baltimore, Md: 1950)* 2011 Aug 15;**187**(4):1856–65.
10. Griese M, Starosta V. Agglutination of Pseudomonas aeruginosa by surfactant protein D. *Pediatric Pulmonology* 2005 Nov 01; **40**(5):378–84.
11. Voorhout WF, Veenendaal T, Kuroki Y, Ogasawara Y, van Golde LM, Geuze HJ. Immunocytochemical localization of surfactant protein D (SP-D) in type II cells, Clara cells, and alveolar macrophages of rat lung. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society* 1992 Oct 01;**40**(10):1589–97.
12. Alten JA, Moran A, Tsimelzon AI, Mastrangelo MA, Hilsenbeck SG, Poli V, et al. Prevention of hypovolemic circulatory collapse by IL-6 activated Stat3. *PLoS One* 2008;**3**(2): e1605.
13. Moran A, Akcan Arikan A, Mastrangelo MA, Wu Y, Yu B, Poli V, et al. Prevention of trauma and hemorrhagic shock-mediated liver apoptosis by activation of stat3alpha. *International Journal of Clinical and Experimental Medicine* 2008;**1**(3):213–47.
14. Moran A, Tsimelzon AI, Mastrangelo MA, Wu Y, Yu B, Hilsenbeck SG, et al. Prevention of trauma/hemorrhagic

shock-induced lung apoptosis by IL-6-mediated activation of Stat3. *Clinical and Translational Science* 2009 Feb;**2**(1):41—9 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
15. Moran A, Thacker SA, Arikan AA, Mastrangelo MA, Wu Y, Yu B, et al. IL-6-mediated activation of Stat3alpha prevents trauma/hemorrhagic shock-induced liver inflammation. *PLoS One* 2011;**6**(6):e21449 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
16. Hierholzer C, Kalff JC, Omert L, Tsukada K, Loeffert JE, Watkins SC, et al. Interleukin-6 production in hemorrhagic shock is accompanied by neutrophil recruitment and lung injury. *American Journal of Physiology* 1998;**275**(3 Pt 1): L611—21.
17. Ono M, Yu B, Hardison EG, Mastrangelo MA, Tweardy DJ. Increased susceptibility to liver injury after hemorrhagic shock in rats chronically fed ethanol: role of nuclear factor-kappa B, interleukin-6, and granulocyte colony-stimulating factor. *Shock* 2004 Jun;**21**(6):519—25.
18. Vanderzwan J, McCaig L, Mehta S, Joseph M, Whitsett J, McCormack DG, et al. Characterizing alterations in the pulmonary surfactant system in a rat model of Pseudomonas aeruginosa pneumonia. *The European Respiratory Journal: Official Journal of the European Society for Clinical Respiratory Physiology* 1998 Dec 01;**12**(6): 1388—96.
19. Webert KE, Vanderzwan J, Duggan M, Scott JA, McCormack DG, Lewis JF, et al. Effects of inhaled nitric oxide in a rat model of Pseudomonas aeruginosa pneumonia. *Critical Care Medicine* 2000 Jul;**28**(7):2397—405 [Research Support, Non-U.S. Gov't].
20. Arikan AA, Yu B, Mastrangelo MA, Tweardy DJ. Interleukin-6 treatment reverses apoptosis and blunts susceptibility to intraperitoneal bacterial challenge following hemorrhagic shock. *Critical Care Medicine* 2006 Mar;**34**(3):771—7 [Evaluation Studies Research Support, N.I.H., Extramural].
21. Crouch E, Persson A, Chang D, Heuser J. Molecular structure of pulmonary surfactant protein D (SP-D). *The Journal of Biological Chemistry* 1994 Jun 24;**269**(25):17311—9 [Research Support, U.S. Gov't, P.H.S.].
22. Mason RJ. Biology of alveolar type II cells. *Respirology (Carlton, Vic)* 2006 Feb;**11 Suppl**:S12—5 [Review].
23. Mason RJ, Williams MC. Type II alveolar cell. Defender of the alveolus. *The American Review of Respiratory Disease* 1977 Jul;**115**(6 Pt 2):81—91.
24. Nayak A, Dodagatta-Marri E, Tsolaki AG, Kishore U. An insight into the diverse roles of surfactant proteins, SP-A and SP-D in innate and adaptive immunity. *Frontiers in Immunology* 2012;**3**:131.
25. Lim BL, Wang JY, Holmskov U, Hoppe HJ, Reid KB. Expression of the carbohydrate recognition domain of lung surfactant protein D and demonstration of its binding to lipopolysaccharides of gram-negative bacteria. *Biochemical and Biophysical Research Communications* 1994 Aug 15; **202**(3):1674—80.
26. Restrepo CI, Dong Q, Savov J, Mariencheck WI, Wright JR. Surfactant protein D stimulates phagocytosis of Pseudomonas aeruginosa by alveolar macrophages. *American Journal of Respiratory Cell and Molecular Biology* 1999 Nov;**21**(5): 576—85.
27. Griese M, Steinecker M, Schumacher S, Braun A, Lohse P, Heinrich S. Children with absent surfactant protein D in bronchoalveolar lavage have more frequently pneumonia. *Pediatr Allergy Immunol* 2008 Nov;**19**(7):639—47.
28. Guzel A, Kanter M, Guzel A, Pergel A, Erboga M. Anti-inflammatory and antioxidant effects of infliximab on acute lung injury in a rat model of intestinal ischemia/reperfusion. *Journal of Molecular Histology* 2012 Jul;**43**(3):361—9.
29. Herbein JF, Wright JR. Enhanced clearance of surfactant protein D during LPS-induced acute inflammation in rat lung. *American Journal of Physiology Lung Cellular and Molecular Physiology* 2001 Jul;**281**(1):L268—77.
30. Cheng IW, Ware LB, Greene KE, Nuckton TJ, Eisner MD, Matthay MA. Prognostic value of surfactant proteins A and D in patients with acute lung injury. *Critical Care Medicine* 2003 Feb;**31**(1):20—7.
31. Jian B, Hsieh C-H, Chen J, Choudhry M, Bland K, Chaudry I, et al. Activation of endoplasmic reticulum stress response following trauma-hemorrhage. *Biochimica et Biophysica Acta* 2008 Nov 01;**1782**(11):621—6.
32. Endo M, Oyadomari S, Suga M, Mori M, Gotoh T. The ER stress pathway involving CHOP is activated in the lungs of LPS-treated mice. *Journal of Biochemistry* 2005 Oct 01;**138**(4): 501—7.
33. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, et al. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes & Development* 1998 May 01;**12**(7):982—95.
34. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, et al. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes & Development* 2004 Dec 15;**18**(24):3066—77 [Comparative Study].
35. Ji C, Mehrian-Shai R, Chan C, Hsu Y-H, Kaplowitz N. Role of CHOP in hepatic apoptosis in the murine model of intragastric ethanol feeding. *Alcoholism, Clinical and Experimental Research* 2005 Aug 01;**29**(8):1496—503.
36. Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in pediatric intensive care units in the United States. National Nosocomial Infections Surveillance System. *Pediatrics* 1999 May 01;**103**(4):e39.
37. Foglia E, Meier MD, Elward A. Ventilator-associated pneumonia in neonatal and pediatric intensive care unit patients. *Clinical Microbiology Reviews* 2007 Jul 01;**20**(3):409—25 [Review]. [table of contents].
38. Sun HY, Fujitani S, Quintiliani R, Yu VL. Pneumonia due to Pseudomonas aeruginosa: part II: antimicrobial resistance, pharmacodynamic concepts, and antibiotic therapy. *Chest* 2011 May;**139**(5):1172—85 [Review].
39. Fujitani S, Sun HY, Yu VL, Weingarten JA. Pneumonia due to Pseudomonas aeruginosa: part I: epidemiology, clinical diagnosis, and source. *Chest* 2011 Apr;**139**(4):909—19 [Review].
40. Grgurich PE, Hudcova J, Lei Y, Sarwar A, Craven DE. Management and prevention of ventilator-associated pneumonia caused by multidrug-resistant pathogens. *Expert Review of Respiratory Medicine* 2012 Nov;**6**(5):533—55 [Review].
41. Brundage SI, Zautke NA, Holcomb JB, Spain DA, Lam JC, Mastrangelo MA, et al. Interleukin-6 infusion blunts proinflammatory cytokine production without causing systematic toxicity in a swine model of uncontrolled hemorrhagic shock. *The Journal of Trauma* 2004 Nov;**57**(5):970—7 [discussion 7—8].
42. Marcu MG, Doyle M, Bertolotti A, Ron D, Hendershot L, Neckers L. Heat shock protein 90 modulates the unfolded protein response by stabilizing IRE1alpha. *Molecular and Cellular Biology* 2002 Dec;**22**(24):8506—13.
43. Gallerne C, Prola A, Lemaire C. Hsp90 inhibition by PU-H71 induces apoptosis through endoplasmic reticulum stress and mitochondrial pathway in cancer cells and overcomes the resistance conferred by Bcl-2. *Biochimica et Biophysica Acta* 2013 Jun;**1833**(6):1356—66.
44. Sinn DI, Chu K, Lee ST, Song EC, Jung KH, Kim EH, et al. Pharmacological induction of heat shock protein exerts neuroprotective effects in experimental intracerebral hemorrhage. *Brain Research* 2007 Mar 2;**1135**(1):167—76 [Research Support, Non-U.S. Gov't].

45. Clark HW. Untapped therapeutic potential of surfactant proteins: is there a case for recombinant SP-D supplementation in neonatal lung disease? *Neonatology* 2010 Jun;**97**(4):380—7 [Review].

46. Evans SE, Scott BL, Clement CG, Larson DT, Kontoyiannis D, Lewis RE, et al. Stimulated innate resistance of lung epithelium protects mice broadly against bacteria and fungi. *American Journal of Respiratory Cell and Molecular Biology* 2010;**42**(1):40—50.