IN VIVO DELIVERY OF CASPASE 8 OR FAS siRNA IMPROVES THE SURVIVAL OF SEPTIC MICE

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ABSTRACT

While studies have shown increased evidence of death receptor-driven apoptosis in intestinal lymphoid cells, splenocytes, and the liver following the onset of polymicrobial sepsis, little is known about the mediators controlling this process or their pathological contribution. We, therefore, attempted to test the hypothesis that the hydrodynamic administration of siRNA against the death receptor, Fas or Caspase 8 should attenuate the onset of morbidity and mortality seen in sepsis, as produced by cecal ligation and puncture (CLP). We initially show that \textit{in vivo} administration of GFP siRNA in GFP transgenic mice results in a decrease in GFP fluorescence in most tissues. Subsequently, we also found that treating septic non-transgenic mice with siRNA targeting Fas or Caspase 8 but not GFP (used as a control here) decreased the mRNA, in a sustained fashion up to 10 days, and protein expression of Fas and Caspase 8, respectively. In addition, TUNEL and active Caspase 3 analyses revealed a decrease in apoptosis in the liver, spleen, but not the thymus following siRNA treatment. Indices of liver damage were also decreased. Finally, the injection of Fas or Caspase 8 given not only thirty minutes but up to twelve hours after CLP significantly improved the survival of septic mice.
INTRODUCTION

Sepsis affects approximately 750,000 people in the United States every year, where one-third of the reported cases result in death. Common causes include traumatic injury, severe bacterial infections, or severe burns. However, sepsis also frequently affects the critically ill, elderly, pediatric, and post-surgical patients in the intensive care unit. With the exception of the recent application of activated protein C, most molecular biological-based therapies have failed clinically. Treatment using antibiotics mildly reduces risk of death, however it is ineffective on mice with severe sepsis, as measured by high inflammatory cytokine levels. Thus, there is an urgent need for not only better understanding of the pathology of sepsis and its resultant organ failure, but a need for new therapeutic approaches and targets.

We and other labs have previously reported that organ damage and mortality associated with sepsis in mouse models is at least in part due to the activation of the Fas-FasL signaling pathway, and not TLR4. Studies using Fas fusion protein (Fas FP) given 12 hours post-CLP show a protective effect against apoptosis in Kupffer cells, which seems to benefit the liver in a way that reduces organ damage and in turn, improves survival after sepsis. This blockade of FasL also restores total hepatic, intestinal, and cardiac blood flow while attenuating the plasma levels of ALT and AST indicating reduced liver damage. In addition, studies using of FasL -/- mice provide further evidence for death receptor-mediated apoptosis in sepsis, as FasL -/- mice also show a marked reduction in septic mortality. Nonetheless, little is known about whether...
truncation of FasL downstream signaling events are protective against septic morbidity and mortality, as few or no studies have examined these signaling targets in sepsis. Pharmacological inhibitors of caspases such as Caspase 8 or Caspase 3 have shown some protection, specifically with pan-specific caspase inhibition. However, significant considerations must be made with caspase peptide inhibitors to avoid not only toxicity, maintain a protective dosage, but to retain caspase specificity. Alternatively, there is a void of inhibitors available for Fas receptor associated proteins which are components of the “death induced signaling complex” (DISC).

With the recent advent of RNAi technology, the blockade of certain genes involved in pathologic mechanisms is being carried out extensively in vitro, but this has rarely been tried in vivo due to the inefficiency of various vectors and their potential inflammatory sequellae. However, utilizing a technique known as hydrodynamic delivery, naked constructs, including small, double-stranded RNAs (siRNA) can be introduced into the tissues of the whole animal by increasing its hydrostatic pressure with a rapid large volume tail vein injection. Recently, a model of ConA/Fas-induced fulminant hepatitis has shown that siRNA targeted against Fas can effectively reduce Fas message when given hydrodynamically (siRNA dissolved in 2 mL volume, injected via the tail vein over 5 seconds), and can maintain a decreased level of the message for up to 10 days. Similarly, siRNA targeted against Caspase 8, a downstream signaling molecule in the Fas pathway has also been used in a FasL-adenovirus mouse model of liver damage and shown to maintain decreased Caspase 8 mRNA and improve survival. However, while these observations are informative, these were largely proof-of-principle experiments in which the mediator/causative mechanism was established, unlike sepsis.
Because the hydrodynamic administration of siRNA is reported to have a prolonged, but not permanent, effect on its target mRNA we employed this technique in the model of polymicrobial sepsis cecal ligation and puncture, CLP, to determine if the blockade of Fas or Caspase 8 attenuates the onset of morbidity and mortality seen in polymicrobial sepsis.

MATERIALS AND METHODS

Animals: The initial gene silencing and tissue distribution studies with siRNA were carried out using female C57BL/6-TgN (ACTbEGFP)/Osb (GFP transgenic) mice (Jackson Laboratories, Bar Harbor, ME). Subsequently, male C3H/HeN (TLR4-deficient, Charles River Laboratories, MA), 6-8 weeks of age were used in Fas and Caspase 8 siRNA experiments. The studies described here were carried out in accordance with the National Institute of Health and The Guide for the Care and Use of Laboratory Animals, and were approved by the Brown University and Rhode Island Hospital Institutional Animal Care and Use Committee.

Model of sepsis: The surgical procedure to generate sepsis was carried out as previously described. C3H/HeN male mice were lightly anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL). The abdomen was shaved and scrubbed with betadine. A midline incision (1.5-2 cm) was made below the diaphragm. The cecum was isolated, ligated, punctured twice with a 22 gauge needle, and was gently compressed to extrude a small amount of cecal material. The cecum was returned to the abdomen, and the muscle and skin incisions were closed with 6-0 Ethilon suture material (ETHICON, INC., Somerville, NJ). Before suturing the skin 2-3 drops of Lidocaine (Abbott Laboratories)
was administered to the wound for analgesia. The mice were subsequently resuscitated with 1.0 mL of Lactated Ringer’s solution subcutaneously. Sham controls were subjected to the same surgical laparotomy and cecal isolation, but the cecum was neither ligated nor punctured.

**Delivery of siRNA:** Fas, Caspase 8, and GFP siRNA was obtained from Dharmacon RNA Technologies (Lafayette, CO). The target sequences used for Fas: 5’-AAGUGCA AGUGCAAAACCAGAC-3’ and Caspase 8: 5’-AACCUCGGGAUACUG UCUGA-3’. The GFP siRNA target sequence was as produced by Dharmacon: 5’- GGCTACGTCCAGGAGCGCACC-3’. For tissue distribution studies, GFP mice were injected with either 2 ml of saline or 50 µg of GFP siRNA dissolved in 2 ml saline.

Twenty-four hours later the mice were killed, and organs harvested for frozen tissue sectioning. Frozen tissues were sectioned on a cryostat (Leico) and visualized using a Nikon Eclipse E400 fluorescent microscope to assess change in GFP intensity after administration of GFP siRNA. The qualitative change in GFP intensity and the overall number of fluorescing cells per field was made by an investigator blinded to the sample identity. Scored as: ++++, highly [50-100% of cells in field fluorescent] fluorescent; ++, moderately fluorescent [25-49%]; +, weakly fluorescent [24-5%]; -, background/non-fluorescent [4-0%].

For the majority of the *in vivo* Fas and Caspase 8 assessment studies, C3H/HeN mice were randomly placed into groups receiving 50 µg of naked Fas, Caspase 8, or GFP siRNA /mouse or 1.5 ml of normal saline alone in the form of a hydrodynamic i.v. injection 30 minutes after CLP. However, in some studies the administration of siRNA was delayed up to 12 hours after CLP. Typically 50 µg of siRNA was dissolved in 1.5
ml PBS and was then injected rapidly (over a period of 5 seconds) into the tail vein. In these studies GFP siRNA was used as a nonsense siRNA control (8). It should be noted that a mouse genome BLAST search indicated the sequence interacted with no known sequence in the mouse.

**Assessment of IFN-α and IL-6 activation:** C3H/HeN mice were injected with either 200 µg of poly I:C (Sigma) to achieve a positive signal, 50 µg of Caspase 8 or Fas siRNA, or were left untreated. The plasma was harvested 18 hours post-injection to analyze circulating levels of IFN-α or IL-6 via a sandwich method ELISA kit (Antigenix America, NY or BD-Pharmingen, respectively) and was carried out according to manufacturer’s instructions. (n=4/group)

**RT-PCR:** For Fas and Caspase 8 mRNA analysis, RNA was isolated from livers of Sham or CLP mice treated with saline, GFP, Fas, or Caspase 8 siRNA. Reverse transcription using an iScript kit (Bio-Rad Laboratories) was performed to generate cDNA. PCR was carried out for 30 cycles using 2.5 µl of cDNA and 22.5 µl PCR Master Mix containing Taq DNA polymerase (Promega, WI). Custom primers were used and are as follows. Fas, AAAGTGGCCCATTTAACAGGC (forward), AAAGCACG GAC AATTCCATAGGTG (reverse). Caspase 8, TGCCCTCAAGTTCCTGTGCTTGGAC (forward), GGATGCTAAGAATGTCATCTCC (reverse). GAPDH, TGCATCCTG CACCACCAACT (forward), AACACGGAAGGCCATGCCAG (reverse). Densitometric analysis was done with an Alpha-Innotek gel documentation station.

**Western Blot:** Protein lysates of mouse liver and spleen were run on 12% Tris-Glycine gels (Invitrogen, Carlsbad, CA), blotting procedures, chemiluminescent detection and densitometric analysis were carried out as previously described by our laboratory.
Membranes were probed with either Fas rabbit polyclonal, Caspase 8 polyclonal antibodies (Santa Cruz, CA), active Caspase 3 (BD Pharmingen) or pro-Caspase 9 (Chemicon).

**Assessment of Apoptosis:** Twenty-four hours post-CLP, mice that had been treated with Fas siRNA, Caspase 8 siRNA, GFP siRNA (nonsense control) (8), or saline were killed to harvest liver and spleen. Both tissues were processed for frozen sectioning. Frozen section slides were fixed with 4% paraformaldehyde and washed with PBS. Slides were incubated with TUNEL reaction mixture according to manufacturer’s protocol (Roche Diagnostics). Degree of apoptosis was assessed by visualizing tissues using a Nikon Eclipse E400 fluorescent microscope. Apoptotic index was derived from the average of the number of TUNEL positive cells vs. the total number of cells in eight random fields at 600X magnification.

**Caspase 3 & 8 activity assays:** As described previously by Chung et al.¹³, livers and splenocytes from septic mice post-treated with saline, GFP, Fas, or Caspase 8 siRNA were homogenized in the presence of lysis buffer containing DTT. 2x reaction buffer containing DTT and AFC-DEVD (Caspase 3) or AFC-IETD (Caspase 8) (BioVision, Mountain View, CA) was added to 50 µg of splenocyte lysate or 400 µg of liver lysate. After 1 hour incubation, samples were read on a fluorescent plate reader (FLx800,Bio-Tek Inc., Winoski, VT) at excitation 400 nm and emission 505 nm and reported the extent of AFC release in arbitrary fluorescent units.

**Plasma Liver Enzyme Levels:** Blood from septic mice post-treated with saline, GFP, Fas, or Caspase 8 siRNA was collected in a syringe containing 2 units of heparin, transferred to a microtube, and centrifuged at 10,000 g for 10 min. at 4° C. Plasma
samples were stored at -80°C until assayed. Plasma AST and ALT levels were determined using a kit (Biotron Diagnostics, Hemet, CA), according to the manufacturer’s instructions.

**Survival Studies:** Approximately thirty minutes following CLP, mice were placed randomly in groups (20-22 mice/group), receiving either saline, GFP siRNA, Fas siRNA, or Caspase 8 siRNA in the form of a hydrodynamic injection. Mice were returned to the animal facility and percent survival was followed and recorded for 10 days.

**Statistics:** The data are presented as a mean and SE of the mean for each group. Differences in percentile (i.e. apoptotic index %) data were considered to be significant at P<0.05, as determined by the Mann-Whitney rank sum test. Changes in ALT/AST levels and IFN-α levels were considered significant at P<0.05, as determined by the unpaired T-test. Results of the survival studies were compared using the chi-square test and were considered significant at P<0.05.

### RESULTS

**Tissue Distribution of siRNA after Hydrodynamic Delivery**

Since *in vivo* use of siRNA has been applied only in mediator defined models specific to the liver, we sought to initially establish the capacity of siRNA to suppress specific (GFP transgene) gene expression and to determine the tissue distribution of siRNA when injected hydrodynamically. To investigate this, GFP transgenic mice were injected hydrodynamically with 50 µg of GFP siRNA, or a saline vehicle. Tissues were then examined using fluorescence microscopy to assess the extent of GFP suppression as a decrease in fluorescence in the siRNA treated animal. We observed that in all the GFP
mouse tissues examined, including spleen and liver (Fig. 1) as well as heart, kidney, lung, muscle, brain, thymus, and Peyer’s patches (data not shown) exhibited a decrease in GFP. This indicates that hydrodynamic administration suppresses, but does not totally ablate, specific gene (GFP) expression as well as provides an effective distribution of siRNA to all major organs/cells.

**Systemic delivery of siRNA does not induce IFN-α and IL-6**

C3H/HeN mice injected with siRNA against Caspase 8 or Fas siRNA alone were analyzed for an increase in interferon activation as typically seen in response to dsRNA. Levels of IFN-α in the plasma of mice treated with siRNA (67 +/- 8 pg/ml) were similar to the level of naïve, untreated mice (33.5 +/- 1.5 pg/ml), as compared (P< 0.05) to the increased levels seen in mice treated with poly I:C (233 +/- 50 pg/ml). IL-6 blood levels exhibited a comparative changes to that of IFN-α, with the levels of about 83.7 +/- 6 pg/ml for naïve, 49.2 +/- 9 pg/ml for siRNA treated animals versus a rise of 3166 +/- 11.2 pg/ml in those mice treated with poly I:C. For each group n=4.

**Hydrodynamic delivery of Fas or Caspase 8 siRNA suppresses respective mRNA and protein expression in septic mice**

Since Fas signaling is up-regulated in sepsis, RT-PCR was used to evaluate the mRNA expression levels of both Fas and Caspase 8 after siRNA treatment post-CLP vs. separate control sets for gene targeted (Fig. 2a & b). As compared to saline and GFP siRNA treated controls, mice treated with Caspase 8 or Fas siRNA show an approximate 40% reduction in the level of Caspase 8 or Fas mRNA in the liver, respectively, comparable to the levels found in the Sham-CLP mouse (Fig. 2a & b). This suppression seems to be
sustained up to day 10 post-CLP, as the hepatic Caspase 8 or Fas expression was still only about 65% off the Sham-CLP group.

In addition, we found that both Caspase 8 or Fas siRNA were able to suppress protein levels of Caspase 8 or Fas in the liver or spleen though day 10 post-injection (Fig. 3a & b). Densitometry analysis showed that the hydrodynamic injection of siRNA suppressed its target mRNA in the liver by ~40% (Fig. 2c) and that specific protein (Fig. 3b & d) expression was alternatively reduced by at least ~40-60%.

**Fas or Caspase 8 siRNA suppresses apoptosis after sepsis in the spleen and liver**

Since numerous studies have previously documented that apoptosis of the immune system, particularly lymphocytes 14 and to a lesser extent myeloid cells 4;15 and non-parenchymal cells, we set out to determine the effect of Fas or Caspase 8 treatment on the onset of septic apoptosis. Fig. 4a illustrates what was typically found following CLP; an increase in the incidence of TUNEL positive cells observed in the CLP mouse liver (Fig. 4.a.i.) and spleen (not shown) which was not affected by treatment with GFP siRNA (Fig. 4.a.ii.). The number of TUNEL positive cells declined in Fas siRNA treated CLP mice (Fig. 4.a.iii.). Quantitation of these changes (Fig. 4b) shows that Fas and Caspase 8 siRNA given after CLP decrease the apoptotic index in the spleen and the liver to sham levels. siRNA treatment did not however, have an effect on apoptosis in the thymus (Fig. 4c). Fig. 5a shows active Caspase 3 levels in the liver and the spleen, confirming the TUNEL findings that Fas or Caspase 8 siRNA treatment after CLP decreases apoptosis. In addition, increased levels of pro-Caspase 9 in the liver (Fig. 5b) and spleen (not shown) after Fas or Caspase 8 siRNA treatment indicate a reduction in apoptosis in these
organs. Lastly, Caspase 3 and 8 activity is decreased with Fas or Caspase 8 treatment (Fig. 5c).

**Fas and Caspase 8 siRNA treatment after CLP decreases plasma liver enzymes**

Earlier work has already provided that blocking Fas decreases the extent of liver damage after sepsis\(^4\);\(^5\). Hepatocellular damage has been reported to occur early during experimental sepsis and is evidenced by an elevation in plasma ALT and AST levels\(^16\);\(^17\). In this respect, we observed that when mice were treated with either Fas or Caspase 8 siRNA, but not GFP siRNA, there was a significant (n=6/group; p<0.05) attenuation of liver enzyme ALT and AST levels of about 30% from the peak levels (28 +/- 3 ALT units/liter and 39 +/- 2 AST units/liter/group) seen in the CLP/saline vehicle group (data not shown).

**Fas and Caspase 8 siRNA treatment improves survival of mice to polymicrobial sepsis**

To the extent that hydrodynamic delivery of siRNAs targeting Fas or other downstream signaling molecules in the pathway (Caspase 8) could provide a survival advantage to septic mice, we initially followed septic mortality of mice receiving siRNA, ~30 min following the performance of CLP, for up to 12 days. In this study, mice injected with Caspase 8 or Fas siRNA after septic insult exhibit a significant increase in survival at 3 days post-CLP and beyond when compared to saline or GFP siRNA controls (Fig. 6a & b).
Delayed administration siRNA against Fas still provides a survival benefit to polymicrobial septic mice

Fig. 6c, inset, illustrates the survival of mice that received a 2 mL hydrodynamic injection of normal saline 12 hours post-CLP as compared to mice that only received the normal 0.8 mL of Lactated Ringer’s solution immediately after CLP. Septic mice receiving an injection 12 hours post-CLP show a transient improvement of survival at 36 hours post-CLP, suggesting that the extra fluids provide some support during the immune hyporesponsive phase of sepsis, and also that the large fluid volume injection is not itself deleterious to the septic mouse. Interestingly, when Fas siRNA suspended in normal saline was included in the resuscitation, given at 12 hours post-CLP a significant survival benefit was still evident (Fig. 6c) in these animals when compared to vehicle treated mice.

DISCUSSION

Our studies have demonstrated that gene transcripts necessary for apoptosis in sepsis can be silenced/suppressed in the septic animal via RNAi. To accomplish this silencing in vivo, siRNA targeting genes of interest can be introduced into the whole animal and reach major organs through a rapid large volume injection. With this in mind, we report that siRNA targeting our genes of interest, Fas and Caspase 8, could effectively be delivered to target organs, those of which are already known to have increased incidence of Fas-driven apoptosis upon septic insult ⁴. Because siRNA was able to decrease Fas and Caspase 8 mRNA and protein levels, it was not surprising that this treatment was also able to decrease apoptosis in the spleen and liver. Although we observed evidence of gene silencing in the thymus, based on the reduction of GFP signal
in the GFP transgenic mice with GFP siRNA, septic thymic apoptosis was not decreased. This, however, is not surprising as we and other labs have shown that the thymus does not seem to undergo Fas-mediated, but undergoes steroid induced apoptosis. To the extent, this decrease in apoptosis prevents injury to the liver. We also noted that treatment with either Fas or Caspase 8 siRNA also decreased plasma liver enzymes, ALT and AST.

From an outcome perspective, we importantly observed that suppression of Fas or Caspase 8 gene expression with this in vivo siRNA administration also conferred a survival advantage to septic mice as compared to controls. Interestingly, this survival benefit was still evident even when the administration of Fas siRNA was provided as late as twelve hours post-CLP. This would suggest that Fas mediated pathologic effects are sustained over-time or require a longer period of time to develop. These findings are also in keeping with the concept that the suppression of genes in this fashion can last 5-10 days based on the reports of others, as well as that seen here. That said, while we found that the suppression of both message and protein production in the liver lasts up to 10 days in our model, we have not explored the nature of this persistence. At present we can only speculate that as cells in the liver under in vivo conditions, which do not divide like cell lines in culture (to which comparisons are made), leads to less dilution of the functional siRNA levels present and thus may allow the silencing effect to persist much longer. This also suggests that protection from Fas activation induced pathology might be comparable at 24 hours post-CLP whether or not the siRNA was given at 30 min or 12 hours post-CLP. This, however, remains to be established in this model.
These results, together with our observation that the loss of caspase 9 pro-form and the increase in caspase 3 and 8 activity in septic mice can be suppressed, further support the role of the extrinsic/Fas-mediated cell death pathway as well as the intrinsic/mitochondrial apoptotic process in the pathology of sepsis. Our findings also demonstrate the potential to target downstream, enzymatic as well as non-enzymatic components of this pathway as possible therapeutic targets. The point being here that as more is understood about the mechanism of RNAi action, its delivery and uptake of siRNA into cells, this method could be modified for use in the clinical setting. Another possible approach is the use of cationic liposomes encoding siRNAs. This technique was used by Sorensen et al. as a pretreatment to endotoxemia. Liposome-based anti-TNF-α siRNAs given intraperitoneally 18 hours prior to LPS injection significantly improved survival of BALB/C mice and decreased TNF-α levels. That said, many gene-based treatments necessitate the use of vectors, many of which cause inflammation and other side effects that minimize their use in inflammatory conditions such as those experienced by the septic animal. Thus, it was a concern that the in vivo infusion of siRNA might induce an inflammatory response through the activation of the interferon pathway. However, we have found that animals treated solely with siRNA did not show an increase in plasma IFN-α levels, which is well documented to rise in response to TLR3, PKR and/or IRF-STAT1 activation by double stranded RNA, while the administration of poly I:C did induce such a change. This would suggest that hydrodynamic delivery of siRNA at this concentration differs distinctly from alternative forms of double or single stranded RNAs as a stimulant of interferon activation.
Using hydrodynamic delivery of plasmid DNA, it has previously been shown that volume and rate of injection are critical in the uptake of naked constructs, in that it must be a large fluid volume (i.e. equivalent of about one time the animal’s blood volume) rapidly injected \(^7\). Liu et al, have shown that the injected transgene can be expressed in the lung, spleen, heart, kidney, and more extensively in the liver \(^7\). Our findings are consistent with Liu et al., even though the construct we used was naked siRNA, we were still able to observe its silencing ability in all major organs after hydrodynamic delivery.

Due to the nature of the hydrodynamic injection, there are obvious clinical limitations with respect to volume and rate given in this way. However, as our main objective here was to document that silencing of upstream and downstream gene targets of an important proapoptotic pathway could be achieved by utilizing a post-treatment siRNA approach in sepsis, the nature/mode of delivery was not our primary concern. Nonetheless, we do appreciate there are attributes of fluid resuscitation in septic patients which need to be considered \(^25; 26\). Here, we explored the effects of hydrodynamic delivery in septic mice and whether a large volume injection of normal saline would cause any untoward effect on survival due to greater resuscitation volume. We found, however, that hydrodynamic fluid delivery had no marked deleterious effects when administered at either 30 min.- 1 hour or 12 hours post-CLP. If anything, there was a transient survival benefit at 36 hours post-CLP which was subsequently lost. It is also important to appreciate that all arms of this study receive hydrodynamic fluid administration. Thus the differences seen would be independent of those effects simply attributable to large volume fluid administration alone.
The question remains, however, as to what cells are targeted by Fas or Caspase 8 siRNA treatment in this model of sepsis that are enabling the animal to survive.

Increased Fas expression is seen on leukocytes, particularly neutrophils and monocytes, of humans treated with endotoxin. After endotoxin treatment, and subsequent systemic inflammation, Fas mRNA expression is increased, indicating de novo transcription and increased surface expression of Fas. This, in turn, increases the susceptibility of target cells to Fas-mediated apoptosis. Not surprisingly, leukocytes of septic patients also exhibit an increase in Fas receptor expression, where higher Fas expression correlates with a higher rate of mortality. A similar phenomenon for FasL has also been recently observed in the bacteremic baboon’s spleen. This is in keeping with the idea that the immune hyporesponsive stage of sepsis contributes to the animal’s inability to defend itself against the lethal polymicrobial challenge. With respect to Fas ligand, there is an increase in the liver after experimental sepsis in mice, but not in the spleen, suggesting that it is the increase in Fas death receptor that is responsible for the increase in apoptosis in these organs. In this respect, our lab has also made a similar finding of increased splenic lymphoid Fas but not FasL gene expression in septic mice. Lymphocytes are also important modulators of the immune response to sepsis. In experimental animals, we see these cells become immunocompromised at the switch from the hyper-responsive to the hypo-responsive stage of sepsis (>12 hours post-CLP), in this model. In patients, many clinically based studies have correlated increased apoptosis, particularly in lymphocytes, with infection, organ dysfunction, and decreased survival of sepsis possibly due to this immunocompromised state. It has been suggested in mouse models that blocking apoptosis of immune cells improves survival and can provide a benefit to
the non-parenchymal cells of an organ such as the liver, as it would prevent bystander cellular damage. In this model, liver, spleen, and thymic cell apoptosis was analyzed by TUNEL and active Caspase 3 expression after treatment with Fas or Caspase 8 siRNA. We see a significant decrease in apoptosis by the decline in TUNEL positive cells, as well as a decrease in active Caspase 3 levels in tissues that usually undergo Fas-mediated apoptosis in sepsis, such as the liver and the spleen. It is possible that this is a “rescue” that is maintaining immune competency in the mouse, thus preventing bystander cellular damage, multiple organ failure, and subsequent morbidity that is normally seen in sepsis. The studies presented here utilize a simple way of introducing siRNA directly into the animal without the use of a vector. In doing so this is an efficacious method for silencing/suppressing genes for apoptosis post-transcriptionally that are normally seen upregulated in the septic state and for that reason are found to contribute to septic mortality from multiple organ failure. Intriguingly, the capacity to alter the septic response in this fashion implies that various downstream components of not only apoptotic, but also non-apoptotic pathways may also be potential targets for this type of siRNA approach. The use of Fas and Caspase 8 siRNA not only identifies these as potential upstream and downstream therapeutic targets in sepsis but also confirms the role of the Fas/FasL signaling pathway in the septic syndrome.
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References


28. Papathanassoglou EDE, Moynihan JA, McDermott MP, Ackerman MH. Expression of Fas (CD95) and Fas ligand on peripheral blood mononuclear cells in critical illness and association with multiorgan dysfunction severity and survival. Crit Care Med. 2001;29:709-718.


FIGURE LEGENDS

**Fig. 1.** Tissue distribution and ability of silencing of GFP siRNA hydrodynamically delivered in GFP transgenic mice, seen at 600X magnification. Suppression of green fluorescent protein was seen in the liver, spleen, thymus, lung, muscle, heart, kidney (shown) brain, and Peyer’s patches (not shown). The qualitative change in GFP intensity and the overall number of fluorescing cells per field was made by an investigator blinded to the sample identity.

**Fig. 2.** Semi-quantitative RT-PCR showing a representative change mRNA levels of Caspase 8 and Fas in the liver relative to GAPDH after respective siRNA treatment (experiment was repeated 3 times). Total RNA was isolated from mice 24 hours, 5 days (d5), or 10 days (d10) after CLP or Sham surgery. Animals that underwent CLP received normal saline, GFP siRNA, Fas siRNA, or Caspase 8 siRNA 30 minutes after surgery. (A) Fas mRNA levels after CLP/Fas siRNA treatment, (B) Caspase 8 mRNA levels after CLP/Caspase siRNA treatment. Levels are decreased to sham levels at 24 hours, 5 days, and 10 days after CLP/Fas or Caspase 8 siRNA treatment. **C,** Densitometry analysis of the intensity of mRNA bands for Fas and Caspase 8 relative to GAPDH.

**Fig. 3.** Protein levels in both the liver and spleen after Fas (A) or Caspase 8 (C) treatment as shown by Western blot, representative of 3 separate repeat experiments. Total protein was isolated from mice 24 hours, 5 days (d5), or 10 days (d10) after CLP or Sham surgery. **B,** Western blot band density (in arbitrary densitometric units, AU) for
Fas and (D) Caspase 8. Black bars represent the liver, open bars represent the spleen. *, P<0.05 vs. CLP/Saline and CLP/GFP siRNA controls, t-test (n=4/group).

**Fig. 4.** Apoptosis in the liver and spleen following siRNA treatment. A, Representative immunofluorescent micrographs indicating TUNEL positive cells in liver sections after CLP. i. Saline treatment, ii. GFP siRNA, and iii. Fas siRNA. B, Apoptotic index of hepatocytes and splenocytes following Caspase 8 and Fas siRNA treatment following CLP as determined by TUNEL. Solid bars, hepatocytes; striped bars, splenocytes. *,# P<.005 vs. Saline and GFP controls, Mann-Whitney U-test (n=5/group). C, Apoptotic index in the thymus following Caspase 8 or Fas siRNA treatment following CLP.

**Fig. 5.** A, Levels of active caspase 3 in the liver and the spleen to confirm TUNEL findings. This blot is representative of 3 repeat experiments. B, Pro-Caspase 9 levels in the liver after siRNA treatment. C, Caspase 3 (top graph) and 8 (bottom graph) activities (in arbitrary fluorescent units) in the liver and the spleen after CLP and Fas or Caspase 8 siRNA treatment.

**Fig. 6 A.** Survival of mice receiving saline, GFP siRNA, or Caspase 8 siRNA 30 min. post-CLP (time of injection indicated by red arrow). Significant survival protection is seen at day 3 *, P<0.05 vs. saline and GFP controls, Chi-square test, (n=20/group). B, Survival of mice receiving saline, GFP siRNA, or Fas siRNA 30 min. post-CLP. Significance in survival is also seen at day 3, *, P<0.05, chi-square test, (n=22/group).
6C, (insert) Percent survival of mice receiving a hydrodynamic injection (2 mL) of normal saline 12 hours post-CLP, as compared to normal resuscitation, 0.8 mL of Lactated Ringer’s immediately following CLP. (6C, Larger graph) The effect of Fas siRNA given hydrodynamically 12 hours after CLP (injection time indicated by red arrow). A significant survival benefit is seen at day 5, *P<0.05 vs. saline and GFP controls, Chi-square test, (n=15-17/group).
Figure 1

Saline  GFP siRNA

Liver
Spleen
Thymus

Saline  GFP siRNA

Lung
Muscle
Heart
Kidney
Figure 2

A. GAPDH
Fas
Sham  Saline  GFP  24h  5d  10d
After siRNA treatment

B. CLP
GAPDH
Caspase 8
Sham  Saline  GFP  24h  5d  10d
After siRNA treatment

C. ratio Fas/GAPDH
ratio Caspase 8/GAPDH
Sham  Saline  GFP  24h  5d  10d
After siRNA treatment
Figure 3

A. Fas Protein

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Liver

Spleen

B.

Liver and Spleen

Levels of Fas protein (AU)

120

100

80

60

40

20

0

CLP: + | - | + | + | + | + | + |
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA:</td>
<td>-</td>
<td>-</td>
<td>GFP</td>
<td>Fas</td>
<td>Fas-d5</td>
<td>Fas-d10</td>
</tr>
</tbody>
</table>

C. Caspase 8 Protein

<table>
<thead>
<tr>
<th>CLP:</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA:</td>
<td>-</td>
<td>-</td>
<td>GFP</td>
<td>C8</td>
<td>C8-d5</td>
<td>C8-d10</td>
</tr>
</tbody>
</table>

Liver

Spleen

D.

Levels of Caspase 8 protein (AU)

140

120

100

80

60

40

20

0

CLP: + | - | + | + | + | + |
<table>
<thead>
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</thead>
<tbody>
<tr>
<td>siRNA:</td>
<td>-</td>
<td>-</td>
<td>GFP</td>
<td>C8</td>
<td>C8-d5</td>
</tr>
</tbody>
</table>
Figure 4

A. TUNEL Expression 24 h post CLP

B. Apoptotic Index (%)

C. Apoptotic Index (%)

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Figure 5

A. **Active Caspase 3 Protein**
   - CLP: + + - + +
   - siRNA: - GFP - Fas Casp 8

   ![Image of Western Blot for Active Caspase 3 Protein]

B. **Pro-Caspase 9 Protein**
   - CLP: + - + + +
   - siRNA: - - GFP Fas Casp 8

   ![Image of Western Blot for Pro-Caspase 9 Protein]

C. **Caspase Activity**

   ![Graph showing caspase activity in liver and spleen]

   - Liver
   - Spleen
Figure 6

A. 

B. 

C. 

Days Post CLP

% Survival

CLP/Fas siRNA
CLP/GFP siRNA
CLP/saline

CLP/Caspase 8 siRNA
CLP/GFP siRNA
CLP/saline

Hrs Post-CLP

Days Post CLP

% Survival

12 Hrs

CLP/Fas siRNA
CLP/GFP siRNA
CLP/saline

CLP
In vivo delivery of Caspase 8 or Fas siRNA improves the survival of septic mice

Doreen E Wesche-Soldato, Chun-Shiang Chung, Joanne Lomas-Neira, Lesley A Doughty, Stephen H Gregory and Alfred Ayala

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