Published in final edited form as:

Crit Care Med. 2016 August ; 44(8): e711–e720. doi:10.1097/CCM.0000000000001672.

Delayed MEK/ERK Inhibition by Trametinib Attenuates Systemic Inflammatory Responses and Multi-Organ Injury in Murine Sepsis

Joshua A. Smith, BS¹, Philip R. Mayeux, PhD², and Rick G. Schnellmann, PhD^{1,3}

¹Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, South Carolina

²Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences

³Ralph H. Johnson Veterans Administration Medical Center, Charleston, South Carolina

Abstract

OBJECTIVE—The MEK/ERK signaling pathway is an essential component of innate immunity necessary for mediating pro-inflammatory responses in the setting of sepsis. We previously demonstrated that the MEK1/2 inhibitor trametinib, prevents endotoxin-induced renal injury in mice. We therefore assessed efficacy of trametinib in a more clinically relevant experimental model of sepsis.

DESIGN—Controlled *in vivo* laboratory study.

SETTING—University animal research laboratory.

SUBJECTS—Male C57BL/6 mice.

INTERVENTIONS—Mice were subjected to cecal ligation and puncture (CLP) to induce sepsis or underwent sham operation as controls. Six hours after CLP, mice were randomized to four experimental groups as follows: 1) sham control; 2) sham control + trametinib (1 mg/kg, i.p.); 3) CLP; and 4) CLP + trametinib. All animals received buprenorphine (0.05 mg/kg, s.c.) and imipenem/cilastatin (14 mg/kg, s.c.) in 1.5 ml of warm saline (40 ml/kg) at the 6 hour time point. Mice were sacrificed at 18 hours after induction of CLP.

MEASUREMENTS AND MAIN RESULTS—Trametinib inhibition of MEK/ERK signaling 6 h after CLP attenuated increases in circulating pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, and GM-CSF) and hypothermia at 18 h. Trametinib also attenuated multi-organ injury as determined by serum creatinine, alanine aminotransferase, lactate dehydrogenase, and creatine kinase. At the organ level, trametinib completely restored peritubular capillary perfusion in the kidney. Restoration of microvascular perfusion was associated with reduced mRNA expression of well-characterized markers of proximal tubule injury. MEK/ERK blockade attenuated CLP-mediated up-regulation of cytokines (TNF- α , IL-1 β) and restored IL-6 to control levels in the renal

cortex, indicating the protective effects on the proximal tubule occur primarily through modulation of the pro-inflammatory response in sepsis.

CONCLUSION—These data reveal that the MEK/ERK inhibitor trametinib attenuates systemic inflammation and multi-organ damage in a clinically relevant model of sepsis. Because trametinib has been safely used in humans, we propose that this drug might represent a translatable approach to limit organ injury in septic patients.

Keywords

sepsis; trametinib; MEK/ERK; multiple organ failure; inflammation

Introduction

Sepsis is a leading cause of morbidity and mortality in the intensive care setting (1). The clinical course in septic patients is often complicated by dysfunction of multiple vital organs including the kidneys, lung, liver, and brain (2). Acute kidney injury (AKI) develops in up to 60% of patients diagnosed with sepsis or septic shock, and septic AKI is associated with an extremely high risk of mortality (~70%) (3). Clinical management of sepsis remains limited to non-specific supportive care aimed at maintaining organ homeostasis and preventing further infection. Although recent initiatives to implement early, goal-directed measures have shown modest benefits in septic patients, mortality resulting from this condition remains unacceptably high (4). Thus, development of novel therapeutics that directly target organ injury and dysfunction remains a top priority in sepsis research.

Organ insult in the setting of sepsis is initiated by activation of pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs), by pathogen-associated molecular patterns (PAMPs) derived from the causative organism(s). Activation of TLRs expressed on immune cells (including dendritic cells, macrophages, neutrophils, and B lymphocytes), endothelial cells, and epithelial cells leads to an overwhelming systemic response characterized by production of acute phase cytokines (TNF- α , IL-1 β , and IL-6), chemokines, and other pro-inflammatory mediators (5). Development of AKI involves combined local and peripheral effects of these factors, which ultimately contribute to microvascular dysfunction and direct tubular injury in the septic kidney (6). The mitogen-activated protein kinase (MAPK) family, including ERK1/2, JNKs, and p38 MAPKs are a major downstream target of TLRs and other PRRs responsible for induction of inflammatory signaling (7, 8).

ERK1/2 are serine/threonine kinases that regulate a variety of cellular processes including survival, differentiation, migration, proliferation, transcription, and metabolism via phosphorylation of a vast array of target proteins. Canonical ERK1/2 signaling is initiated by binding of growth factors (VEGF, IGF-1, EGF) to their respective receptor tyrosine kinases (RTKs) leading to downstream activation of the Ras/Raf/MEK/ERK pathway. However, more recent evidence indicates that other cellular signaling entities, including TLRs, also activate ERK1/2. TLR4 stimulation by lipopolysaccharide (LPS), a critical mediator of gram-negative sepsis, increases ERK1/2 activity in macrophages by engaging the tumor progression locus-2 (TPL-2)/mitogen activated protein kinase kinase-1/2 (MEK1/2)/ERK1/2 cascade. ERK1/2 activation in this context plays a critical role in

mediating the immune response to PAMPs by increasing transcription and translation of pro-inflammatory cytokines (TNF- α , IL-1 β) and chemokines (CXCL2) in multiple cell types (9, 10). Despite considerable evidence linking MEK/ERK signaling to innate immunity, studies investigating this pathway as a potential therapeutic target for prevention of unchecked inflammation and organ dysfunction in the setting of sepsis are lacking.

We recently demonstrated that the FDA-approved MEK1/2 inhibitor trametinib (also known as GSK1120212 or JTP-74057) attenuates renal injury following systemic LPS exposure in mice (11). Pharmacological blockade of MEK/ERK signaling reversed endotoxin-induced increases in blood urea nitrogen (BUN) and mRNA expression of kidney injury molecule-1 (KIM-1), a marker of proximal tubule injury, at both 3 and 18 h time points. Restoration of renal function following trametinib administration was associated with decreased expression of TNF- α and IL-1 β transcripts in the renal cortex, further suggesting that MEK/ERK signaling may regulate organ-specific inflammatory responses in this model.

A subsequent study by Shi-Lin et al. showed that trametinib significantly inhibits LPS-induced TNF- α production in mouse primary bone marrow-derived macrophages and human peripheral blood mononuclear cells (PBMCs) (12). In addition, MEK/ERK inhibition prevented mortality resulting from co-administration of LPS and the sensitizing agent D-galactosamine in mice in association with decreased levels of circulating TNF- α (12). Since trametinib has been safely used in the clinic, these findings suggest that targeted MEK/ERK inhibition may have translational potential as a novel therapy for sepsis-induced AKI and organ dysfunction in humans (13). It should be noted that clinical relevance of endotoxin models in rodents is limited due to a number of differences from human sepsis. Most importantly, systemic LPS exposure in mice leads to a massive, transient increase in serum pro-inflammatory cytokine levels (TNF- α , IL-1 β , IL-6) whereas elevations in these cytokines are more protracted and several orders of magnitude lower in septic humans (14). In addition, therapies that have shown promise in experimental endotoxin models including inhibitors of TNF- α and IL-1 have largely failed to improve mortality and other secondary outcome measures in clinical trials for sepsis likely due in part to the differences noted above.

The purpose of the current study was to evaluate efficacy of the MEK/ERK inhibitor trametinib in a clinically relevant model of sepsis induced by cecal ligation and puncture (CLP) in mice. We demonstrate here that post-treatment with trametinib at 6 h following CLP attenuated the systemic inflammatory response and development of hypothermia. Trametinib administration also prevented development of AKI and multiple organ injury in CLP animals. In the kidney, MEK/ERK inhibition restored microvascular perfusion and reduced expression of tubular injury markers including kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and heme oxygenase-1 (HO-1) at the mRNA level (15-18). The renoprotective effects of trametinib were associated with decreased renal expression of pro-inflammatory cytokines including TNF- α and IL-1 β and a return of IL-6 to control levels. Taken together, our data provide evidence that the FDA-approved MEK/ERK inhibitor trametinib may be a viable drug to attenuate systemic inflammatory responses and organ injury in the setting of sepsis.

Materials and Methods

Mouse Cecal Ligation and Puncture Model of Sepsis

Male C57BL/6 mice aged 40 weeks were obtained from Harlan Laboratories (Indianapolis, IN). Cecal ligation and puncture was performed as previously described (19, 20). Briefly, 1.5 cm of the cecum was mobilized and ligated with a 4-0 silk suture under isoflurane anesthesia after midline laparotomy. The mobilized cecum was then punctured with a 21-gauge needle and gently pressed to produce a ~1 mm column of fecal material from each puncture site. Sham animals underwent midline laparotomy followed by isolation of the cecum, but the cecum was not ligated or punctured. All mice received buprenorphine (0.05 mg/kg, s.c.) at the time of surgery. At 6 h post-CLP mice received a second dose of buprenorphine and imipenem/cilastatin (14 mg/kg, s.c.) in 1.5 ml of warm saline for fluid support (40 ml/kg). Mice were euthanized 18 h after CLP and kidneys and serum were collected for analysis. Core body temperature was measured using a rectal temperature probe. All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use was approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Administration of Trametinib

Trametinib (Selleckchem, Houston, TX) was formulated in vehicle solution containing 1% DMSO in Neobee M-5 (Spectrum Chemical, New Brunswick, NJ) (21). Trametinib is a specific and potent allosteric inhibitor of MEK1/2 signaling that has been used in animal models and humans (13, 22). Mice were treated with 1 mg/kg trametinib or an equal volume of vehicle solution via intraperitoneal (i.p.) injection at 6 h after CLP.

Determination of Circulating Cytokine Levels

Measurement of serum levels of TNF- α , IL-1 β , IL-6 and GM-CSF was performed using the Inflammatory Cytokine Mouse Magnetic 4-Plex Panel for the Luminex platform (Life Technologies, Grand Island, NY). Detection of cytokines was performed using a Bio-Rad Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA) and quantified using a standard curve and protein standards supplied in the kit. For values that fell below the lower limit of detection of the assay in sham animals, values were recorded as the lower limit (TNF- α – 22.4 pg/ml; IL-1 β – 28.1 pg/ml; IL-6 – 9.8 pg/ml; GM-CSF – 16.06 pg/ml).

Intravital Microscopy and Assessment of Peritubular Capillary Perfusion

To assess changes in renal microcirculation after CLP, intravital video microscopy was performed as previously described (20, 23). Mice were anesthetized with isoflurane and FITC-labeled dextran (2 μ mol/kg in 3 ml/kg normal saline) was injected via the penile vein to visualize microvascular flow. After 10 minutes, the left kidney was exposed by a flank incision and placed on a glass stage above an inverted Zeiss Axiovert 200M fluorescent microscope with an AxioCam HSM camera (Zeiss, Jena, Germany). Ten second videos were acquired at ~30 frames per second from five randomly selected, non-overlapping fields for each animal. Core body temperature was maintained at 35-37°C with a heating lamp.

Roughly 150 capillaries from each animal were selected for analysis and categorized into three categories based on the degree of perfusion as follows: “continuous flow” where red blood cell movement was uninterrupted throughout the video; “intermittent flow” where red blood cell movement stopped or reversed during the course of the video; or “no flow” where no red blood cell movement was observed throughout the video. Data are shown as percentage (mean $\pm$ S.E.M.) of vessels in each category.

Serum Chemistries

Blood urea nitrogen (BUN) was measured using the QuantiChrom Urea Assay kit as directed by the manufacturer (BioAssay Systems, Hayward, CA). Serum creatinine (SCr) was assessed using the Creatinine Enzymatic Reagent Set based on the provided protocol (Pointe Scientific, Canton, MI). Alanine aminotransferase (ALT), creatine kinase (CK), and lactate dehydrogenase (LDH) were measured using standard biochemical assay kits from BioVision (Milpitas, CA) as directed.

Quantitative Real-Time PCR Analysis of Gene Expression

Total RNA was extracted from renal cortex tissue samples using the TRIzol reagent (Life Technologies). One microgram of total RNA was reversed transcribed into a cDNA library using the iScript Advanced cDNA Synthesis Kit for quantitative real-time polymerase chain reaction (qRT-PCR) based on the manufacturer's protocol (Bio-Rad). qRT-PCR was then performed with the synthesized cDNA using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Relative mRNA content for all genes of interest was calculated by the 2^{-C_t} method as previously described using β -actin as a reference for normalization (24). Primer sequences used for qRT-PCR were the same as previously reported (11, 25).

Immunoblot Analysis

Protein was isolated from the renal cortex in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4) containing protease inhibitor cocktail (1:100), 1 mM sodium fluoride, and 1 mM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO). Protein quantification was performed using the BCA protein assay. Equal quantities of protein were loaded onto 4-15% SDS-PAGE gels (Bio-Rad) and resolved by gel electrophoresis. Primary antibodies used for this study included phospho-ERK1/2 (Thr202/Tyr204, 1:1000) and total ERK1/2 (1:1000, Cell Signaling Technology, Danvers, MA), and β -Actin (1:1000, Santa Cruz Biotechnology, Dallas TX). Proteins of interest were imaged using enhanced chemiluminescence (ECL) reagents and the GE ImageQuant LAS4000 digital imaging system (GE Life Sciences, Pittsburgh, PA). Optical densitometry was performed using NIH ImageJ software (version 1.46).

Statistical Analysis

All data are presented as mean $\pm$ S.E.M. A one-way analysis of variance (ANOVA) followed by Neuman-Keuls post hoc test was performed for statistical comparisons. A *P* value less than 0.05 was considered statistically significant. All statistical tests were completed using GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

Delayed Trametinib Administration Decreases CLP-induced Systemic Inflammatory Cytokine Production and Hypothermia

Because MEK/ERK signaling is thought to be necessary for generation of pro-inflammatory cytokines in response to innate immune system activation in sepsis, we determined the effects of trametinib on the systemic inflammatory response following CLP by measuring TNF- α , IL-1 β , IL-6, and GM-CSF (12). Serum levels of TNF- α were below the lower limit of detection (22.4 pg/ml) in sham animals, but rose after CLP (~400 pg/ml). Interestingly, post-treatment with trametinib at 6 h after CLP induction attenuated (~50%) the increase in TNF- α at 18 h post-CLP (Figure 1 A). In a similar manner, serum levels of IL-1 β , IL-6, and GM-CSF were elevated in mice subjected to CLP and trametinib partially reduced these circulating pro-inflammatory cytokines (Figure 1 B,C,D). It should be noted that all animals survived at 6 hours post-CLP. However, by the 18 hour time point, only 8/11 (73%) of the CLP + vehicle animals survived. In contrast, 8/8 animals (100%) in the CLP + trametinib group survived.

In addition, we measured core body temperature as a secondary index of the systemic response. Development of hypothermia is strongly correlated with physiological dysfunction and mortality in the CLP model (26). At 6 h after CLP, both CLP + vehicle and CLP + trametinib animals had markedly reduced core body temperatures (~7°C) in comparison to vehicle-treated sham controls (Figure 1 E). Trametinib alone did not have a significant effect on body temperature at 18 h in sham animals. However, treatment with trametinib completely attenuated CLP-induced hypothermia (Figure 1 E). Taken together, these data are consistent with the idea that trametinib treatment prevents progression of the systemic inflammatory response following CLP in mice.

Trametinib Post-treatment Partially Attenuates AKI and Multiple Organ Injury After CLP

Given that MEK/ERK inhibition effectively reduced the systemic inflammatory response, we assessed the effects of trametinib on organ/cellular damage following CLP. SCr and BUN, used as measures of renal dysfunction, increased 2-fold and 4-fold, respectively 18 h post-CLP when compared to sham controls (Figure 2 A,B). Trametinib treatment at 6 h completely reversed the CLP-induced rise in SCr, but had no effect on BUN (Figure 2 A,B). In addition, we measured serum markers of hepatic (ALT), skeletal muscle/myocardial (CK), and general cellular damage (LDH) to further evaluate the efficacy of trametinib in sepsis. CLP resulted in elevations in ALT (~5.4-fold), CK (~6.7-fold), and LDH (~4-fold) at 18 h (Figure 2 C,D,E). These findings are consistent with previous reports of multi-organ injury following CLP in mice (19, 27). Importantly, delayed trametinib administration partially attenuated increases in hepatic (ALT), skeletal muscle/myocardial (CK), and cellular damage (LDH) after CLP (Figure 2 C,D,E). These findings reveal that MEK/ERK blockade decreases injury to multiple organs in the setting of sepsis.

Delayed Trametinib Administration Blocks CLP-induced ERK Activation in the Renal Cortex

To further elucidate the mechanisms by which trametinib protects organ integrity after CLP, we focused on changes in the kidney. Immunoblot analysis in renal cortical lysates confirmed that trametinib effectively inhibits MEK/ERK signaling after CLP. An increase in phosphorylated/activated ERK1/2 (~2-fold) was observed in vehicle-treated CLP animals (Figure 3 A,B). Phospho-ERK levels were decreased in CLP + trametinib mice when compared to both CLP and sham conditions 12 h after drug administration (Figure 3 A,B). In addition, trametinib administration diminished ERK activation in sham mice (Figure 3 A,B).

Delayed Trametinib Administration Attenuates Inflammatory Changes in the Renal Cortex After CLP

Direct effects of pro-inflammatory cytokines (TNF- α , IL-1 β) on the proximal tubule play an important role in development of sepsis-induced AKI (11, 28). Thus, we determined if trametinib-mediated attenuation of AKI in the CLP model is associated with decreased pro-inflammatory cytokine expression in the renal cortex. Transcript levels of TNF- α , IL-1 β , and IL-6 increased in the renal cortex in CLP animals (Figure 4 A,B,C). Trametinib either fully (TNF- α) or partially (IL-1 β , IL-6) attenuated these changes (Figure 4 A,B,C). These data reveal that MEK/ERK signaling is essential for up-regulation of pro-inflammatory cytokines in the renal cortex following CLP and may be associated with proximal tubule injury in this model.

Effects of Delayed MEK/ERK Inhibition on Renal Peritubular Capillary Perfusion and Tubular Injury Following CLP

Microvascular dysfunction is a common contributor to sepsis-induced organ injury, including AKI, and is thought to occur secondary to cytokine-induced disruption of endothelial cell integrity (29). In particular, a marked reduction in renal peritubular capillary perfusion has been noted in multiple experimental models of sepsis in association with development of local areas of hypoxia and oxidative stress (23, 29, 30). Using intravital video microscopy, a decrease in the portion of peritubular capillaries with continuous red blood cell flow was noted in mice subjected to CLP along with a concomitant increase in capillaries classified by continuous or no flow (Figure 5 A). Trametinib administration at 6 h post-CLP completely reversed the deficiencies in peritubular capillary perfusion described above (Figure 5 A). Representative videos can be found in supplemental videos S1, S2 and S3. These data indicate that MEK/ERK inhibition by trametinib protects the renal microvasculature and improves perfusion after CLP and in mice.

We next investigated the effects of trametinib on renal proximal tubule cell injury. Expression of well-characterized markers of tubular insult including KIM-1, NGAL, and HO-1 were assessed in the renal cortex by qPCR (15-18, 31). When compared to sham controls, a robust increase in KIM-1 mRNA content (~83-fold) was observed in the renal cortex following CLP (Figure 5 B). Treatment of CLP animals with trametinib reduced KIM-1 transcript levels (Figure 5 B). Likewise, delayed MEK/ERK inhibition reduced CLP-mediated induction of NGAL and HO-1 (Figure 5 C,D). Taken together, these findings

reveal that restoration of renal microvascular perfusion by trametinib is associated with a reduction in renal proximal tubular injury following sepsis-induced AKI.

Discussion

Sepsis is defined by the Surviving Sepsis Campaign Guidelines Committee in 2012 as the probable or documented presence of infection accompanied by systemic manifestations of infection (32). Severe sepsis is further defined as sepsis plus sepsis-induced organ dysfunction or tissue hypoperfusion (32). Clinically, sepsis is associated with significant mortality and morbidity characterized by injury to multiple organ systems (2, 27, 30). Innate immune system activation in the setting of sepsis is largely mediated through activation of PRRs by microbial products collectively referred to as pathogen-associated molecular patterns (PAMPs). Stimulation of PRRs initiates signaling through a variety of downstream targets that act to promote the pro-inflammatory response including multiple MAPK signaling pathways involving MEK/ERK, JNK, and p38 MAPKs (7, 8).

Although members of the JNK and p38 MAPK families have classically been implicated in the host response to infection, more recent evidence suggests that MEK/ERK signaling may control multiple aspects of the pro-inflammatory state associated with sepsis. Exposure of macrophages to lipopolysaccharide (LPS), a classical TLR4 agonist, has been shown to activate the MEK/ERK cascade *in vitro* (9, 12). MEK/ERK signaling is required for transcriptional up-regulation of TNF- α and IL-1 β in certain populations of LPS-treated macrophages (9, 12). In addition, ERK activation in response to LPS in macrophages promotes nucleocytoplasmic transport and subsequent translation of TNF- α mRNA, indicating that the MEK/ERK signaling pathway may facilitate TNF- α biosynthesis by both transcriptional and post-transcriptional mechanisms (10). The molecular machinery necessary for stimulation of the MEK/ERK cascade in response to LPS includes TLR4 and its downstream effector TPL-2, a MAP kinase kinase kinase (MAP3K) that directly phosphorylates and activates MEK. Interestingly, TPL-2 is essential for endotoxin-induced TNF- α production in macrophages, and TPL-2-deficient mice are significantly protected against lethality following systemic administration of LPS and D-galactosamine (10). These data suggest that the MEK/ERK signaling pathway represents a novel and viable therapeutic target for the treatment of sepsis and sepsis-induced organ dysfunction. Unfortunately, studies that directly target MEK/ERK in animal models of sepsis are lacking.

We recently used the potent and specific MEK1/2 inhibitor trametinib in a mouse model of endotoxin-induced AKI. Administration of trametinib (1 mg/kg, i.p.) inhibited LPS-induced phosphorylation of ERK1/2 in the renal cortex, indicating that *in vivo* administration of low dose trametinib is sufficient to block this signaling cascade in the LPS model (11). Trametinib pre-treatment at 1 h before systemic LPS exposure in mice attenuated early (< 3 h) development of renal dysfunction as measured by BUN. Preservation of renal function by MEK/ERK blockade was associated with reduced expression of KIM-1, a well-characterized marker of proximal tubule injury, and pro-inflammatory cytokines (TNF- α , IL-1 β) in the renal cortex of LPS-treated mice (11). A subsequent study demonstrated that MEK/ERK inhibition by trametinib also blocked TNF- α production in macrophage cell lines and isolated human PBMCs exposed to LPS *in vitro* (12). Trametinib treatment also prevented

death of mice exposed to LPS and D-galactosamine in association with a reduction in circulating levels of TNF- α (12).

Trametinib is a highly potent allosteric inhibitor with IC₅₀ values of 0.92 nM and 1.8 nM for MEK1 and MEK2, respectively in an *in vitro* protein kinase assay (22). This compound also displays high specificity for MEK1/2, with no significant inhibitory activity seen on a panel of over 100 kinases including other members of the MAPK family (22). Trametinib has been safely and effectively used in humans and recently received FDA approval as a chemotherapeutic agent for use in BRAF-mutant melanoma (13). Thus, this drug has a number of favorable properties that make it an ideal candidate for clinical use in sepsis. However, the endotoxin models used to date to evaluate efficacy of trametinib suffer from a number of severe limitations. Both studies delivered this compound either before or concurrent with LPS administration, a scenario which would not be feasible in the clinical setting. In addition, neither study employed supportive measures commonly used in the intensive care unit such as fluid replacement, broad-spectrum antibiotics, and/or vasopressor agents (33). It should also be noted that the inflammatory response to LPS in mice is much more rapid and of a higher magnitude than that observed in septic humans (14). These fundamental differences likely explain why therapies developed using endotoxin models (such as anti-TNF- α agents) have largely failed to improve outcomes in septic patients (34). Thus, we sought to determine the efficacy of trametinib in attenuating systemic inflammatory responses and organ injury in a more clinically relevant model of sepsis.

We employed a well-established sepsis model induced by cecal ligation and puncture (CLP) in mice. CLP is widely considered the “gold standard” of sepsis models because it produces a much lower grade and sustained pro-inflammatory response when compared to LPS models (35). The model used here in 40-week-old mice also incorporates supportive measures including a broad-spectrum β -lactam antibiotic (imipenem/cilastatin) and fluids (0.9% saline, 40 ml/kg, s.c.) at 6 hours post-CLP to better mimic the basic clinical support of the elderly population, which is commonly affected by sepsis (1, 33). In addition, we chose to delay trametinib administration until 6 h post-CLP, a time when organ injury and microvascular dysfunction is already established (19, 23, 27).

Delayed inhibition of MEK/ERK signaling attenuated CLP-induced increases in all pro-inflammatory cytokines measured including TNF- α , IL-1 β , IL-6, and GM-CSF. Among these, the most significant inhibitory effect was observed on TNF- α . These findings are consistent with earlier studies demonstrating that MEK/ERK signaling is required for maximal induction of TNF- α synthesis in immune cells exposed to TLR agonists (7, 10, 12). Interestingly, serum levels of TNF- α , IL-1 β , and IL-6 may all serve as reliable predictors of mortality resulting from sepsis in the clinical setting, and IL-6 appears to be the single best predictor of poor outcomes in septic patients (36, 37). In addition, IL-6 has been validated as a marker of disease burden following CLP in mice (38). The observed decreases in circulating pro-inflammatory cytokines in CLP animals treated with trametinib likely indicate reduced disease severity. These findings suggest that MEK/ERK inhibition may prevent CLP-induced mortality, although we did not assess this outcome. In support of this, trametinib also reversed development of hypothermia, a widely used indicator of physiologic dysfunction and impending death in septic mice (26).

Reducing systemic pro-inflammatory cytokines alone may have significant effects on organ dysfunction in the setting of sepsis. Cunningham et al. elegantly demonstrated that LPS-induced AKI is mediated by TNF- α acting on TNF receptor 1 (TNFR1) expressed on renal cells (28). Administration of a single dose of recombinant TNF- α is sufficient to cause AKI and subsequent renal dysfunction in rodents (11). Similar effects of pro-inflammatory cytokines have also been described in other organ systems that commonly become dysfunctional in sepsis including the liver and the lung (39, 40). Thus, it is important to note that the systemic effects of MEK/ERK inhibition may be beneficial in preventing injury and dysfunction in multiple organ systems.

As discussed above, multiple organ dysfunction syndrome (MODS) is a common consequence of sepsis, and injury to multiple organs including the kidney, lungs, liver, and skeletal/cardiac muscle contributes to morbidity and mortality resulting from this disease. Our previous study demonstrated that trametinib attenuates early changes in renal dysfunction and proximal tubular injury after endotoxin administration in mice. We therefore examined organ/cellular injury in the CLP model to better demonstrate efficacy of this drug in sepsis. Delayed trametinib administration reversed renal dysfunction as assessed by changes in SCr after CLP. However, there was no change in BUN in trametinib-treated animals. Reasons for the observed discrepancy in SCr and BUN are unclear. It should be noted that BUN can be altered by a number of extrarenal factors in sepsis including increased protein catabolism, glucocorticoid production, and extracellular fluid volume (41). In addition, the change in BUN after CLP may also reflect increased tubular reabsorption of urea rather than glomerular filtration rate (GFR). Trametinib also had protective effects on clinical markers of injury to the liver (ALT), skeletal/cardiac muscle (CK), and general cellular injury (LDH). Taken together, these findings are consistent with the idea that MEK/ERK inhibition by trametinib may be effective in treating multiple organ system injury in sepsis. However, it should be noted that a significant limitation of the current study is that we did not study lung and liver injury in detail. Future studies are warranted to further determine the effects of trametinib on CLP-induced injury to these organs.

For the remainder of our studies, we focused on CLP-induced AKI. The pathophysiology of sepsis-induced AKI and is complex and multi-factorial in nature. Increased endothelial cell permeability in the renal microvasculature leads to sluggish peritubular capillary flow, development of local areas of hypoxia and hypoperfusion, and increased exposure of the renal parenchyma to circulating pro-inflammatory molecules due to reduced flow (6, 42, 43). There is now strong experimental evidence indicating that therapeutic targeting of renal microvascular dysfunction may reverse sepsis-induced AKI (20, 44, 45). As expected, CLP reduced the percentage of peritubular capillaries with continuous flow, and this phenomenon occurred in conjunction with an increase in the proportion of vessels characterized by intermittent or no flow. Treatment with trametinib completely restored renal microvascular perfusion after CLP. These findings are in agreement with earlier studies demonstrating that MEK/ERK signaling plays an important role in development of increased endothelial cell permeability and dysfunction in response to inflammatory insult *in vitro* (46).

Restoration of renal microvascular perfusion following treatment with trametinib was associated with a reduction in transcriptional up-regulation of multiple markers of tubular

injury. In particular, we observed substantial increases in mRNA expression of KIM-1, NGAL, and HO-1 in renal cortical tissue at 18 h after CLP, which is consistent with previous reports (47). Trametinib treatment partially attenuated changes in each of these markers of tubular injury. These findings are in agreement with our previous studies employing MEK/ERK inhibition in the LPS model and suggest that inhibition of MEK/ERK signaling may partially prevent insult to the renal proximal tubule following sepsis-induced AKI (11).

In our previous study, pre-treatment with trametinib partially attenuated local increases in inflammatory cytokine (TNF- α , IL-1 β) production in the renal cortex after systemic endotoxin exposure in mice (11). Trametinib fully attenuated CLP-induced up-regulation of TNF- α in the renal cortex. Similar effects, though less pronounced, were also noted on renal transcript levels of IL-1 β and IL-6. Taken together, these data suggest that MEK/ERK inhibition prevents local, organ-specific inflammatory responses in the setting of sepsis. In support of this, previous reports have observed a reduction in tissue levels of TNF- α , IL-1 β , and IL-6 in the lung and liver in CLP mice following treatment with the MEK1 inhibitor PD98059 (48). It is likely that attenuation of both systemic and local cytokine production contributes to trametinib-mediated protection of the proximal tubule after sepsis-induced AKI. Indeed, treatments aimed at reducing renal inflammation have proven beneficial in experimental models of AKI (49, 50). It should also be noted that oxidative stress and reperfusion injury may play an important role in sepsis-induced AKI. A limitation of the current study is that we did not investigate the effects of trametinib on oxidative stress and reperfusion injury in the CLP model (23).

In summary, we have demonstrated here that a potent, specific allosteric inhibitor of MEK1/2, trametinib, may reduce the systemic inflammatory response and partially attenuate AKI and other organ injury in a clinically relevant model of sepsis induced by CLP in mice. Given the favorable pharmacological properties of trametinib and its recent FDA approval, we believe that MEK/ERK inhibition by trametinib may represent a readily translatable approach to limit organ injury in the setting of sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources: This work was supported in part by National Institute of General Medical Sciences [GM084147 (to R.G.S.), GM106419 (to P.R.M.) and P20GM103542-02 (to South Carolina COBRE in Oxidants, Redox Balance, and Stress Signaling)]; National Center for Research Resources [UL1-RR029882]; the Biomedical Laboratory Research and Development Program of the Department of Veterans Affairs [5101 BX-000851 (to R.G.S.)], and the South Carolina Clinical and Translational Research Institute at the Medical University of South Carolina. Animal facilities were funded by National Center for Research Resources [C06-RR015455].

Copyright form disclosures: Dr. Schnellmann received support for article research from the National Institutes of Health (NIH). His institution received funding from the National Institutes of Health, National Institute of General Medical Sciences, and the Biomedical Laboratory Research and Development Program of the Department of Veterans Affairs. Dr. Smith received support for article research from the NIH. His institution received grant support from the National Institutes of Health, National Institute of General Medical Sciences, and Biomedical Laboratory Research and Development Program of the Department of Veterans Affairs. Dr. Mayeux received support for article research from the NIH and received funding from the Journal of Pharmacology and Experimental Therapeutics (Associate Editor). His institution received grant support from the NIH R01 GM106419.

References

1. Angus DC, Linde-Zwirble WT, Lidicker J, et al. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Critical care medicine*. 2001; 29(7): 1303–1310. [PubMed: 11445675]
2. Singh S, Evans TW. Organ dysfunction during sepsis. *Intensive care medicine*. 2006; 32(3):349–360. [PubMed: 16474954]
3. Schrier RW, Wang W. Acute renal failure and sepsis. *The New England journal of medicine*. 2004; 351(2):159–169. [PubMed: 15247356]
4. Levy MM, Rhodes A, Phillips GS, et al. Surviving sepsis campaign: association between performance metrics and outcomes in a 7.5-year study. *Critical care medicine*. 2015; 43(1):3–12. [PubMed: 25275252]
5. Zhang B, Ramesh G, Uematsu S, et al. TLR4 signaling mediates inflammation and tissue injury in nephrotoxicity. *Journal of the American Society of Nephrology : JASN*. 2008; 19(5):923–932. [PubMed: 18256356]
6. Gomez H, Ince C, De Backer D, et al. A unified theory of sepsis-induced acute kidney injury: inflammation, microcirculatory dysfunction, bioenergetics, and the tubular cell adaptation to injury. *Shock*. 2014; 41(1):3–11. [PubMed: 24346647]
7. Arthur JS, Ley SC. Mitogen-activated protein kinases in innate immunity. *Nature reviews Immunology*. 2013; 13(9):679–692.
8. Jiang R, Ye J, Zhu B, et al. Roles of TLR3 and RIG-I in mediating the inflammatory response in mouse microglia following Japanese encephalitis virus infection. *Journal of immunology research*. 2014; 2014:787023. [PubMed: 25101306]
9. Reimann T, Buscher D, Hipskind RA, et al. Lipopolysaccharide induces activation of the Raf-1/MAP kinase pathway. A putative role for Raf-1 in the induction of the IL-1 beta and the TNF-alpha genes. *Journal of immunology*. 1994; 153(12):5740–5749.
10. Dumitru CD, Ceci JD, Tsatsanis C, et al. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell*. 2000; 103(7):1071–1083. [PubMed: 11163183]
11. Smith JA, Stallons LJ, Collier JB, et al. Suppression of mitochondrial biogenesis through toll-like receptor 4-dependent mitogen-activated protein kinase/extracellular signal-regulated kinase signaling in endotoxin-induced acute kidney injury. *The Journal of pharmacology and experimental therapeutics*. 2015; 352(2):346–357. [PubMed: 25503387]
12. Shi-Lin D, Yuan X, Zhan S, et al. Trametinib, a novel MEK kinase inhibitor, suppresses lipopolysaccharide-induced tumor necrosis factor (TNF)-alpha production and endotoxin shock. *Biochemical and biophysical research communications*. 2015; 458(3):667–673. [PubMed: 25684183]
13. Johnson AS, Crandall H, Dahlman K, et al. Preliminary Results from a Prospective Trial of Preoperative Combined BRAF and MEK-Targeted Therapy in Advanced BRAF Mutation-Positive Melanoma. *Journal of the American College of Surgeons*. 2015; 220(4):581–593. e581. [PubMed: 25797743]
14. Remick DG, Ward PA. Evaluation of endotoxin models for the study of sepsis. *Shock*. 2005; 24(Suppl 1):7–11. [PubMed: 16374366]
15. Zager RA, Johnson AC, Becker K. Plasma and urinary heme oxygenase-1 in AKI. *Journal of the American Society of Nephrology : JASN*. 2012; 23(6):1048–1057. [PubMed: 22440905]
16. Mishra J, Ma Q, Prada A, et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *Journal of the American Society of Nephrology : JASN*. 2003; 14(10):2534–2543. [PubMed: 14514731]
17. Ichimura T, Bonventre JV, Bailly V, et al. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *The Journal of biological chemistry*. 1998; 273(7):4135–4142. [PubMed: 9461608]
18. Adler M, Ramm S, Hafner M, et al. A Quantitative Approach to Screen for Nephrotoxic Compounds In Vitro. *Journal of the American Society of Nephrology : JASN*. 2015

19. Miyaji T, Hu X, Yuen PS, et al. Ethyl pyruvate decreases sepsis-induced acute renal failure and multiple organ damage in aged mice. *Kidney international*. 2003; 64(5):1620–1631. [PubMed: 14531793]
20. Wang Z, Sims CR, Patil NK, et al. Pharmacologic targeting of sphingosine-1-phosphate receptor 1 improves the renal microcirculation during sepsis in the mouse. *The Journal of pharmacology and experimental therapeutics*. 2015; 352(1):61–66. [PubMed: 25355645]
21. Hippalgaonkar K, Majumdar S, Kansara V. Injectable lipid emulsions-advancements, opportunities and challenges. *AAPS PharmSciTech*. 2010; 11(4):1526–1540. [PubMed: 20976577]
22. Yamaguchi T, Kakefuda R, Tajima N, et al. Antitumor activities of JTP-74057 (GSK1120212), a novel MEK1/2 inhibitor, on colorectal cancer cell lines in vitro and in vivo. *International journal of oncology*. 2011; 39(1):23–31. [PubMed: 21523318]
23. Wang Z, Holthoff JH, Seely KA, et al. Development of oxidative stress in the peritubular capillary microenvironment mediates sepsis-induced renal microcirculatory failure and acute kidney injury. *The American journal of pathology*. 2012; 180(2):505–516. [PubMed: 22119717]
24. Wills LP, Trager RE, Beeson GC, et al. The beta2-adrenoceptor agonist formoterol stimulates mitochondrial biogenesis. *The Journal of pharmacology and experimental therapeutics*. 2012; 342(1):106–118. [PubMed: 22490378]
25. Stallons LJ, Whitaker RM, Schnellmann RG. Suppressed mitochondrial biogenesis in folic acid-induced acute kidney injury and early fibrosis. *Toxicology letters*. 2014; 224(3):326–332. [PubMed: 24275386]
26. Ebong SJ, Goyert SM, Nemzek JA, et al. Critical role of CD14 for production of proinflammatory cytokines and cytokine inhibitors during sepsis with failure to alter morbidity or mortality. *Infection and immunity*. 2001; 69(4):2099–2106. [PubMed: 11254563]
27. Dear JW, Yasuda H, Hu X, et al. Sepsis-induced organ failure is mediated by different pathways in the kidney and liver: acute renal failure is dependent on MyD88 but not renal cell apoptosis. *Kidney international*. 2006; 69(5):832–836. [PubMed: 16518342]
28. Cunningham PN, Dyanov HM, Park P, et al. Acute renal failure in endotoxemia is caused by TNF acting directly on TNF receptor-1 in kidney. *Journal of immunology*. 2002; 168(11):5817–5823.
29. Verma SK, Molitoris BA. Renal Endothelial Injury and Microvascular Dysfunction in Acute Kidney Injury. *Seminars in nephrology*. 2015; 35(1):96–107. [PubMed: 25795503]
30. Vincent JL, De Backer D. Microvascular dysfunction as a cause of organ dysfunction in severe sepsis. *Critical care*. 2005; 9(Suppl 4):S9–12. [PubMed: 16168075]
31. Nath KA, Balla G, Vercellotti GM, et al. Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *The Journal of clinical investigation*. 1992; 90(1):267–270. [PubMed: 1634613]
32. Dellinger RP, Levy MM, Rhodes A, et al. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. *Critical care medicine*. 2013; 41(2):580–637. [PubMed: 23353941]
33. Doi K, Leelahavanichkul A, Yuen PS, et al. Animal models of sepsis and sepsis-induced kidney injury. *The Journal of clinical investigation*. 2009; 119(10):2868–2878. [PubMed: 19805915]
34. Dyson A, Singer M. Animal models of sepsis: why does preclinical efficacy fail to translate to the clinical setting? *Critical care medicine*. 2009; 37(1 Suppl):S30–37. [PubMed: 19104223]
35. Buras JA, Holzmann B, Sitkovsky M. Animal models of sepsis: setting the stage. *Nature reviews Drug discovery*. 2005; 4(10):854–865. [PubMed: 16224456]
36. Casey LC, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Annals of internal medicine*. 1993; 119(8):771–778. [PubMed: 8379598]
37. Hack CE, De Groot ER, Felt-Bersma RJ, et al. Increased plasma levels of interleukin-6 in sepsis. *Blood*. 1989; 74(5):1704–1710. [PubMed: 2790194]
38. Remick DG, Bolgos G, Copeland S, et al. Role of interleukin-6 in mortality from and physiologic response to sepsis. *Infection and immunity*. 2005; 73(5):2751–2757. [PubMed: 15845478]
39. Nessler N, Launey Y, Aninat C, et al. Clinical review: The liver in sepsis. *Critical care*. 2012; 16(5):235. [PubMed: 23134597]

40. Ware LB. Pathophysiology of acute lung injury and the acute respiratory distress syndrome. *Seminars in respiratory and critical care medicine*. 2006; 27(4):337–349. [PubMed: 16909368]
41. Beier K, Eppanapally S, Bazick HS, et al. Elevation of blood urea nitrogen is predictive of long-term mortality in critically ill patients independent of “normal” creatinine. *Critical care medicine*. 2011; 39(2):305–313. [PubMed: 21099426]
42. Wu L, Gokden N, Mayeux PR. Evidence for the role of reactive nitrogen species in polymicrobial sepsis-induced renal peritubular capillary dysfunction and tubular injury. *Journal of the American Society of Nephrology : JASN*. 2007; 18(6):1807–1815. [PubMed: 17494883]
43. Wu L, Tiwari MM, Messer KJ, et al. Peritubular capillary dysfunction and renal tubular epithelial cell stress following lipopolysaccharide administration in mice. *American journal of physiology Renal physiology*. 2007; 292(1):F261–268. [PubMed: 16926442]
44. Holthoff JH, Wang Z, Patil NK, et al. Rolipram improves renal perfusion and function during sepsis in the mouse. *The Journal of pharmacology and experimental therapeutics*. 2013; 347(2): 357–364. [PubMed: 24018639]
45. Holthoff JH, Wang Z, Seely KA, et al. Resveratrol improves renal microcirculation, protects the tubular epithelium, and prolongs survival in a mouse model of sepsis-induced acute kidney injury. *Kidney international*. 2012; 81(4):370–378. [PubMed: 21975863]
46. Varma S, Breslin JW, Lal BK, et al. p42/44MAPK regulates baseline permeability and cGMP-induced hyperpermeability in endothelial cells. *Microvascular research*. 2002; 63(2):172–178. [PubMed: 11866540]
47. Zou L, Feng Y, Li Y, et al. Complement factor B is the downstream effector of TLRs and plays an important role in a mouse model of severe sepsis. *Journal of immunology*. 2013; 191(11):5625–5635.
48. Zhang H, Mochhala SM, Bhatia M. Endogenous hydrogen sulfide regulates inflammatory response by activating the ERK pathway in polymicrobial sepsis. *Journal of immunology*. 2008; 181(6):4320–4331.
49. Ramesh G, Reeves WB. Salicylate reduces cisplatin nephrotoxicity by inhibition of tumor necrosis factor-alpha. *Kidney international*. 2004; 65(2):490–499. [PubMed: 14717919]
50. Chen YY, Yeh CH, So EC, et al. Anticancer drug 2-methoxyestradiol protects against renal ischemia/reperfusion injury by reducing inflammatory cytokines expression. *BioMed research international*. 2014; 2014:431524. [PubMed: 25229058]

Figure 1. Delayed trametinib administration decreases CLP-induced systemic responses
Male C57BL/6 mice were treated with the MEK/ERK inhibitor GSK1120212 (1 mg/kg, i.p.) at 6 h post-CLP or sham surgery and euthanized 18 hr after CLP. Serum was collected and circulating levels of the pro-inflammatory cytokines TNF- α (A), IL-1 β (B), IL-6 (C), and GM-CSF (D) were determined using a multiplex Luminex assay. For animals that fell below the lower limit of detection of the assay in sham animals, values were recorded as equal to the lower limit (TNF- α – 22.4 pg/ml; IL-1 β – 28.1 pg/ml; IL-6 – 9.8 pg/ml; GM-CSF – 16.06 pg/ml). Core body temperature was also monitored via a rectal temperature probe (E). Data are expressed as mean $\pm$ S.E.M. for each group. Different superscripts above bars indicate statistically significant differences (p < 0.05; sham + vehicle, n = 6; sham + trametinib, n = 6; CLP + vehicle, n = 7; CLP + trametinib, n = 8).

Figure 2. Trametinib post-treatment decreases CLP-induced organ injury

Serum creatinine (**A**), blood urea nitrogen (**B**), alanine aminotransferase (**C**), creatine kinase (**D**), and lactate dehydrogenase (**E**) were assessed at 18 h after CLP or sham surgery as indicators of organ and cellular injury. Data are shown as mean $\pm$ S.E.M. for each group (sham + vehicle, n = 6; sham + trametinib, n = 6; CLP + vehicle, n = 5; CLP + trametinib, n = 8). Different superscripts above bars indicate significant differences between groups ($p < 0.05$).

Figure 3. Delayed trametinib administration effectively blocks ERK1/2 activation in the renal cortex after CLP

Mice undergoing CLP or sham operation were treated with trametinib (1 mg/kg, i.p.) or vehicle control and kidneys were collected at 18 h. Immunoblot analysis of phosphorylated ERK1/2 (Thr202/Tyr204) (**A,B**) in the renal cortex revealed complete inhibition of MEK/ERK signaling in trametinib-treated animals. Levels of phospho-ERK1/2 were normalized to total ERK1/2 content and actin was used as a secondary loading control. Data are presented as phosphorylated / total ratio relative to vehicle controls (mean $\pm$ S.E.M.; (sham + vehicle, n = 6; sham + trametinib, n = 6; CLP + vehicle, n = 6; CLP + trametinib, n = 6). Different superscripts above indicate statistically significant differences ($p < 0.05$) between groups.

Figure 4. Trametinib post-treatment attenuates expression of pro-inflammatory cytokines in the renal cortex after CLP

Inflammatory changes were evaluated in the renal cortex of CLP mice treated with trametinib (1 mg/kg, i.p.). qPCR was used to determine mRNA levels of TNF- α (A), IL-1 β (B) and IL-6 (C) at 18 h post-CLP. Values are expressed as relative expression vs. control (mean $\pm$ S.E.M.). Superscripts above bars indicate statistically significant differences between groups ($p < 0.05$; sham + vehicle, $n = 6$; sham + trametinib, $n = 6$; CLP + vehicle, $n = 6$; CLP + trametinib, $n = 7$). The superscript “a,b” indicates that this group is not significantly different from control or CLP + vehicle groups.

Figure 5. Trametinib post-treatment restores peritubular capillary perfusion and reduces tubular injury after CLP

Peritubular capillary perfusion was assessed by intravital video microscopy (IVVM) after CLP as described above. The observed microvasculature was classified based on degree of perfusion as having “continuous,” “intermittent,” or “no flow.” Trametinib completely reversed CLP-induced deficiencies in peritubular capillary perfusion (**A**). Data are expressed as percentage of peritubular capillaries in each category (mean $\pm$ S.E.M.; sham + vehicle, $n = 6$; sham + trametinib, $n = 6$; CLP + vehicle, $n = 6$; CLP + trametinib, $n = 7$). Transcript levels of markers of proximal tubular injury including KIM-1 (**B**), NGAL (**C**), and HO-1 (**D**) in the renal cortex were determined by qPCR analysis. Data are shown as fold change relative to sham + vehicle animals (mean $\pm$ S.E.M.; sham + vehicle, $n = 6$; sham + trametinib, $n = 6$; CLP + vehicle, $n = 6$; CLP + trametinib, $n = 7$). Superscripts above bars indicate statistically significant differences ($p < 0.05$, $n = 5$ /group).