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Original Article

Nephrilin peptide modulates a neuroimmune stress response in rodent models of burn trauma and sepsis

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Abstract: Sepsis occurs three times more often in burns than in other types of trauma, suggesting an overlap or synergy between underlying immune mechanisms in burn trauma and sepsis. Nephrilin peptide, a designed inhibitor of mTORC2, has previously been shown to modulate a neuroimmune stress response in rodent models of xenobiotic and metabolic stress. Here we investigate the effect of nephrilin peptide administration in different rodent models of burn trauma and sepsis. In a rat scald burn model, daily subcutaneous bolus injection of 4 mg/kg nephrilin significantly reduced the elevation of kidney tissue substance P, S100A9 gene expression, PMN infiltration and plasma inflammatory markers in the acute phase, while suppressing plasma CCL2 and insulin C-peptide, kidney p66shc-S36 phosphorylation and PKC-beta and CGRP in dorsal root ganglia at 14 days (chronic phase). In the mouse cecal ligation and puncture model of sepsis, nephrilin fully protected mice from mortality between surgery and day 7, compared to 67% mortality in saline-treated animals, while significantly reducing elevated CCL2 in plasma. mTORC2 may modulate important neuroimmune responses in both burn trauma and sepsis.

Keywords: Nephrilin, p66shc, S100A9, neuroimmune, burns, sepsis

Introduction

Traumatic stress is associated with secondary complications such as sepsis and organ failure, which lead to morbidity and death [1, 2]. Burn trauma patients experience a 3-fold higher incidence of sepsis than other trauma patients as well as higher mortality associated with sepsis, suggesting some overlap in the underlying biology of sepsis and burn trauma [3, 4]. The leading cause of death in severely burned children is sepsis [5]. If sepsis and burn trauma do indeed share some underlying process, inhibiting a molecular target involved in that process should reduce both sepsis mortality as well as burn injury morbidity.

Nephrilin, a 40-mer peptide designed as a competitive inhibitor of mammalian target of rapamycin complex 2 (mTORC2) binding to PRR5/Protor, has previously been shown to modulate the neuroimmune response to a variety of stressors in rodents [6, 7]. mTOR levels in dorsal root ganglia are elevated by peripheral inflammation [4]. mTORC2 is a highly conserved kinase complex in mammalian cells, important in the maturation of AGC family kinases such as Akt, PKC and SGK [8-10]. When injected into mice at high doses daily for 26 days, nephrilin generates no visibly differential pathology compared to vehicle [6]. If burn trauma and sepsis do indeed involve aspects of the same neuroimmune stress response (NSR) then nephrilin administration should alleviate both septic mortality and the chronic sequelae of burn injury.

Rodent models of burn trauma and of sepsis do not completely recapitulate the human neuroimmune response in either case, each being optimized to model only some window of it. Burn sepsis models are not optimized to measure the chronic metabolic effects of burn trauma, which are important in human burn patients, focusing instead on short-term mortality.

Table 1. Sequences of peptides and oligonucleotides

A. Amino acid sequence of nephrilin peptide.			
Ac-RGVTEDYLRLETLVQKVVSKGFYKKKQCRPSKGRKRGFCW-amide			
B. Nucleotide sequences of primers used in the PCR.			
Gene	Bases	Forward Primer	Reverse Primer
GAPDH	107	5' GGGCTCTCTGCTCCTCCCTGTT 3'	5' ACGGCCAAATCCGTTCACACCG 3'
S100A9	113	5' ATCATGGAGGACCTGGACAC 3'	5' GGGTTGTTCTCATGCAGCTT 3'

To identify a common underlying mechanism between burns and sepsis, the use of two different models - one optimized for measuring the chronic effects of scald injury, the other for measuring acute sepsis mortality - is required.

In this study we use two models: a rat scald model [11] optimized for low (<1%) mortality, thus allowing measurement of chronic neuroimmune and metabolic abnormalities, and the mouse cecal puncture and ligation (CLP) model [12, 13] which allows measurements of differential septic mortality over a 7-day period. As the type of stress in the CLP model (surgical) is unrelated to burn trauma, it allows us to investigate the question of overlapping mechanisms from the more precise perspective of the molecular target involved, rather than the type of stress. Since nephrilin is a targeted peptide inhibitor, the unbiased logical triangulation of this approach reduces the possibility that the observed treatment effects would somehow be idiosyncratic to the way burn stress is experimentally administered rather than indicative of an overlap in the underlying biology.

In these models, we plan to track three important components of the NSR: (a) *a neurogenic component* canonically involving changes in protein kinase C beta (PKC-β-II) status and transient receptor potential cation channel V (TRPV) family signaling in the dorsal root ganglia [14-17]; these changes are believed to cause activation of neurokinin receptor and peripheral release of neuropeptides such as pro-inflammatory Substance P (SP) and calcitonin-gene-related peptide (CGRP, an immunosuppressive neuropeptide) from sensory neurons into the tissues [18-21]. Models of burn sepsis recapitulate acute inflammatory and delayed immunosuppressive effects that may be monitored via circulating cytokines [22-24]. Of special significance are the CCL-2/MCP-1-mediated actions of polymorphonuclear leukocyte (PMN)-II neutrophils to suppress M1Mphi in favor of M2mphi macrophages [25-29]. Quantitative immunological detection of PKC-β-II, SP, CGRP, IL-6, TNF-alpha, IL-10, IL-33, cathepsin G and CCL-2 are used in this study to track these neuroimmune processes; (b) *oxidative damage* involving p66shc-regulated mitochondrial reactive oxygen species (ROS) generation. The active form of the p66shc adaptor is phosphorylated by PKC-β-II at Serine 36 [30, 31]. Immunological detection of the phosphorylated form of p66shc as a marker of mitochondrial ROS in kidney tissue has been used in a previous study [7]. In kidney, tubular epithelial cells are known to be exquisitely sensitive to oxidative damage [32]; (c) *metabolic dysregulation* consequent to severe burn injury may include post-burn elevation of insulin resistance, endogenous catecholamines and cortisol; and these perturbations may persist for years [1, 33-36]. Key molecules believed to underlie the insulin resistant state in this context include IL-6, phospho-p66shc-S36 and S100A9, a ligand of RAGE [37-40]. In the rat scald model, S100A9 gene expression is dramatically elevated in liver [41]. Plasma insulin C-peptide has previously been employed as a convenient marker of post-burn hypermetabolic abnormality [42]. We use these markers to investigate the effect of nephrilin in rodent models of burn trauma and sepsis.

Materials and methods

Reagents

Nephrilin peptide, a 40-mer peptide carrying a sequence derived from PRR5/Protor (the sequence is conserved in human, rat and mouse species) was synthesized by Genemed Synthesis (San Antonio, TX) and purified to >80% purity by HPLC. The design and synthesis of nephrilin have been previously described [6]. The sequence of the peptide is shown in **Table 1**. BCA Protein Kit was from Pierce (Rockford, IL). Antibodies for ELISAs were purchased from

Santa Cruz Biotechnology (Santa Cruz, CA) except for phospho-p66shc-S36, (Abcam, Cambridge, MA). Sandwich ELISA kits for IL-6, TNF-alpha, IL-10 and IL-33 were purchased from R&D Systems (Minneapolis, MN). CelLytic M cell lysis reagent was obtained from Sigma (St. Louis, MO).

Nephrilin administration

In each rodent model, nephrilin was administered once daily by subcutaneous bolus at 4 mg/kg, with the first dose administered as soon as practicable after completion of surgical or scald procedure. Injection volume was 400 µl for rats and 100 µl for mice. Control animals received the same volume of saline. The 4 mg/kg daily dosage of nephrilin was selected based on its demonstrated safety and efficacy in seven different rodent models tested to date [6]. In a non-GLP study, mice treated daily with 20 mg/kg nephrilin by subcutaneous bolus for 26 days showed no differential toxicology in major organs when compared to a saline control [6].

Rat scald model

The rat scald burn model [11] is a modified Walker-Mason model that induces inflammation and hypermetabolism in line with what severely burned patients experience. The model results in a mortality rate of <1%. Adult male Sprague Dawley rats (200-225 gm-Charles River, USA) were housed in clean plastic cages on a 12 hr light/dark cycle with access to food (standard chow) and water *ad libitum.* Animals were allowed to acclimate for one week prior to the experiment. All animal procedures were performed in adherence to the National Institute of Health's *Guide for Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch at Galveston. All procedures were initiated in the morning between 7 and 10 a.m. Prophylactic analgesia (0.05 mg/kg body weight Buprenorphin) was administered 5 min before general anesthesia (40 mg/kg body weight ketamine/xylazine). The dorsum of the trunk and the abdomen were shaved, and a 60% of total body surface area (TBSA) burn administered by placing the animals in a mold and immersing them in 98-100°C water for 10 seconds on the back and for 2 seconds on the abdomen. This method delivers a full-thickness cutaneous burn as confirmed by histological examination. Burned rats were immediately resuscitated with 40 cc/kg Ringer's Lactate injected intraperitoneally. Sham group was treated exactly as described above for burned animals except that the animals were not placed in hot water. Animals were randomly assigned to treatment groups and nephrilin (4 mg/kg) or saline were administered by subcutaneous bolus daily. Each treatment group comprised 5-6 animals per time point. At the end of the study period animals were sacrificed by cervical dislocation as approved by the University of Texas Medical Branch IACUC guidelines, the NIH's Office of Laboratory Animal Welfare (OLAW), and the AVMA recommendations. Animals were sacrificed on days 1 and 14. Following sacrifice, all organs of interest were rapidly dissected and flash frozen in liquid nitrogen with subsequent storage at -80°C or fixed in formalin and then embedded in paraffin until analysis. Dissection of dorsal root ganglia (DRG) was carried out under the supervision of an experienced specialist in the technique. Briefly, thoracicolumbar DRG were dissected under a dissecting microscope with the ventral side facing down. Two pairs of forceps were used to gently open the skin and muscle around the spine with care not to damage the spinal column area. The connective tissue was removed carefully to clearly expose the spinal column, the thoracolumbar DRGs, and spinal nerves on both sides. The thoracolumbar DRGs were dissected using two pairs of fine-tip forceps and immediately frozen with powdered dry ice until analysis. Tissue integrity was confirmed by microscopy. Plasma TNF-α and IL-6, as well as kidney tissue SP were measured using kits from R&D Systems (Minneapolis, MN). Frozen tissue slices were used for RNA extraction, gene array and qPCR for GAPDH, actin and S100A9 genes. Differences between treatment groups were evaluated using Student's *t*-test ($p<0.05$) for comparing effects on various markers.

Cecal ligation model

All procedures were approved by the Institutional Animal Care and Use Committee of New York University School of Medicine and complied with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male C57BL/6 mice

used for this study were commercially obtained (Taconic Labs, Hudson, NY) and were between 8-12 weeks of age and had a mean weight of 27.14 grams. Mice were acclimated to the housing suite for 1 week prior to the experiment and had access to food and water *ad libitium* and were on a 12:12 L/D lighting cycle. Animals were housed in disposable plastic cages lined with sterile corn cob bedding. Each housing unit contained 4-5 individuals. Cages mates were not separated during the course of the study. Mice were given access to food and water *ad libitium* throughout the duration of the experiment. Generally, welfare of animals and intervention guidelines were provided by AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. For subject termination following induction of septicemia, in consultation with the institutional veterinarian, we assigned termination of subject criteria as: severely hunched posture, reduced body temperature, weight loss (>10% of starting weight), inappetence, moribund state, inability move in order to obtain feed or water. Mice were initially anesthetized using 5% vaporized isoflurane and maintained at 1% throughout the surgical procedure. The abdomen was shaved and disinfected prior to surgery with betadine solution and 70% ethanol. A lateral incision was made through two layers and the cecum exposed and ligated at 0.75 cm from the most distal region of the cecum. Two perforations were made side by side, with a 21.5 gauge needle. After perforation, the ligated portion of the cecum was gently squeezed to expose a small amount of fecal matter. Cecum was returned to the abdominal cavity and abdomen was closed in two layers, using 4-0 braided silk sutures to close the inner peritoneal cavity and 7 mm stainless steel wound clips to close the outer skin layer. Animals were resuscitated on a heating pad set to 26°C. Four sham mice were operated on in the same manner, exposing the cecum but no ligation or perforation was made. All surgical procedures were performed after 12 PM but not after 5 PM each day. Before the animal was fully aroused post surgery, 1 mL of 37°C prewarmed sterile saline solutions was injected subcutaneously on the animals' dorsal side. Upon arousal, each animal was given a subcutaneous injection of the analgesic, buprenorphine, at 0.05 mg/kg of body weight. Mice were randomly selected to be given one of two treatments (group n=6): nephrilin at 4 mg/kg of body weight or sterile saline solution. Group sizes were derived from post-hoc power analyses from studies of similar design. Animals were allocated to experimental groups randomly and by cage assignment. All individuals in a single cage were either assigned treatment or vehicle. Treatments were delivered to mice by subcutaneous bolus daily by researcher blind to treatment identity. 24 hours post-operative, blood was collected from each animal from the tail vein into a capillary blood collection tube coated with EDTA. Blood was also collected at the time of death via cardiac puncture, with the exception of mice found dead over night, where blood collection was not possible. Plasma separation was done in a centrifuge at 700rpm for 25 mins and immediately stored at -80°C. One kidney was collected per animal at the time of death and immediately stored at -80°C. Percent mortality per group was expressed as a 3-day moving average.

Cell extracts, fractionation, ELISAs and immunoprecipitation

Preparation of tissue cell extracts, cell fractionation, and ELISAs were performed as previously described [33, 34]. In order to permit more precise quantitation, specific immunoreactivity was measured by ELISA, rather than by Western Blot.

Immunohistochemistry

Immunohistochemical staining of rat kidney was performed for phosphorylated p66shc-S36. Five-micron sections from formalin fixed paraffin embedded tissues were de-paraffinized with xylenes and rehydrated through a graded alcohol series. Heat induced epitope retrieval was performed by immersing the tissue sections at 98°C for 20 minutes in 10 mM citrate buffer (pH 6.0) with 0.05% Tween. Slides were treated with 3% hydrogen peroxide and 10% normal horse serum and exposed to primary antibodies for p66shc-S36 (1:50, Abcam, ab54518) overnight at 4°C. Slides were exposed to horse anti-mouse rat absorbed biotin-conjugated secondary antibody (Vector Labs) and Cy3 conjugated streptavidin (Zymed) to detect p66shc-S36. Slides were mounted with ProLong Antifade Gold with DAPI (Invitrogen) for nuclear counterstain. Consecutive sections with the omitted primary antibody were used as negative controls. Images were

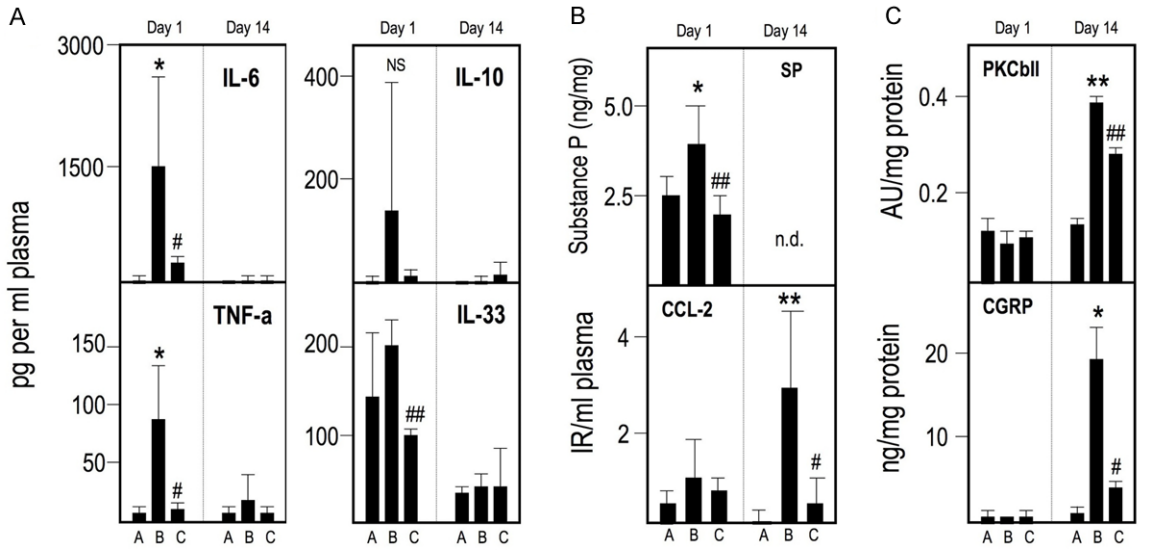

Figure 1. Rat scald model: markers of the NSR. A=sham group (n=6); B=burn+saline group (n=6); C=burn+nephrilin group (n=6); Panel A: Plasma markers; Panel B: Top, substance P in kidney tissue; bottom, CCL2/MCP-1 ELISA immunoreactivity (arbitrary units/ml plasma); Panel C: PKC and CGRP ELISA immunoreactivity in DRG extracts; *p<0.05 **p<0.01 (group A versus B); #p<0.05 ##p<0.01 (group B versus group C); AU=arbitrary units.

Table 2. Cathepsin G in kidney tissue at 24 hours post-burn

	Cathepsin G (U/mg protein)	*p* value
Sham	4.4±0.5	
Burn+Saline	5.7±1.0	0.0332[a]
Burn+Nephrilin	3.2±0.4	0.0011[b]

[a]versus sham group; [b]versus saline group.

captured using an Olympus BX61 Spinning disc confocal microscope at 100x magnification.

RNA extraction

30-50 mg of tissue was homogenized in TRIzol® (Invitrogen, Carlsbad CA) and RNA extracted according to the manufacturer's protocol. Linear Acrylamide (AMRESCO, Solon OH) was added as a co-precipitant at a final concentration of 25 µg/mL. Concentration and purity of the RNA was determined using the NanoDrop spectrophotometer (Thermo Scientific, Wilmington DE).

Quantitative PCR

Quantitative polymerase chain reaction (qPCR) for S100A9 gene transcripts were performed using RNA extracted from kidney tissue and expressed relative to transcripts of GAPDH, a housekeeping gene. S100A9 (previously known as MRP8/14) is a RAGE ligand highly induced in liver transcripts of burned animals in the rat scald model [41]. In preliminary experiments using this model we showed that the S100A9 transcript was highly induced in bladder, kidney, lung and spleen tissues (results not shown). The cDNA synthesis reaction was carried out using 1,000 nanograms of RNA in a final volume of 20 µl following manufacturer's instructions (High-Capacity cDNA Reverse Transcription kit, Applied Biosystems, CA). qPCR was carried out with the Fast Real Time 7500 (Applied Biosystems). The final reaction volume was 20 µl and contained: each primer at a final concentration of 200 nM, Power Sybr Green (Applied Biosystems) 1X and 2 µl of template (dilution 1:4 of cDNA synthesis reaction). Samples and standards were run in triplicate. Primer sequences for detecting S100A9 gene transcripts (S100A9L-S100A9R) and control primers for targeting the messenger RNA for control housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) are shown in **Table 1**. The thermo cycle conditions were as follows: one cycle at 50°C for 20 sec, one activation cycle at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 45 seconds at 60°C. Melting curve analysis was carried out using the continuous method from the 7500 Software (Applied Biosystems) conducted at

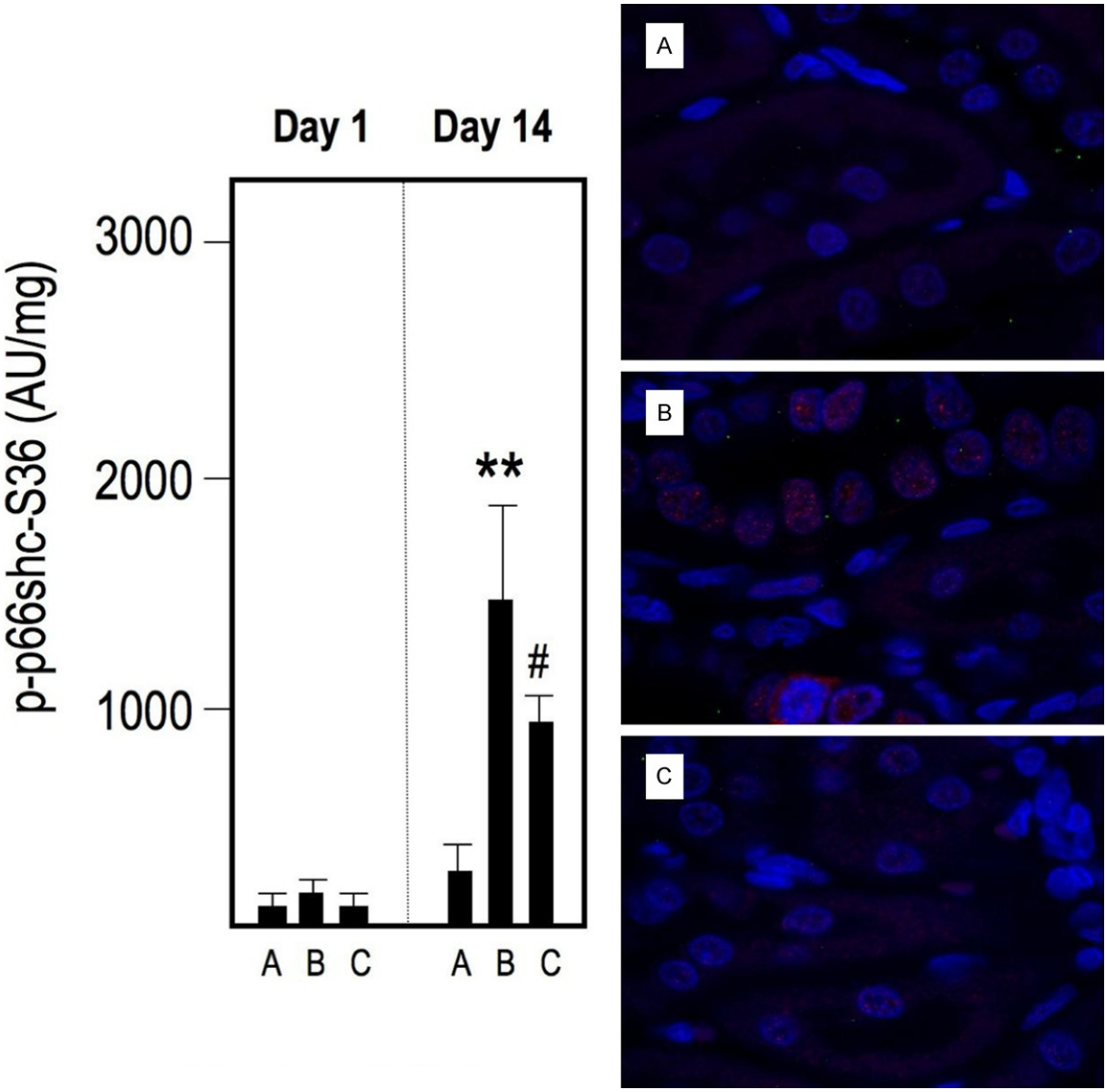

Figure 2. Rat scald model: mitochondrial ROS in kidney tissue. A=sham group (n=6); B=burn+saline group (n=6); C=burn+nephrilin group (n=6); Left Panel: Phospho-p66shc-S36 ELISA immunoreactivity in kidney tissue extracts; Right Panel: Immunohistochemistry of kidney tissue slices using anti-phospho-p66shc-S36 antibody: Slides were exposed to primary antibodies for p66shc-S36 (1:50, Abcam, ab54518) overnight at 4°C as described in Methods. Images were captured using an Olympus BX61 Spinning disc confocal microscope at 100x magnification; *p<0.05 **p<0.01 (group A versus B); #p<0.05 ##p<0.01 (group B versus group C); AU=arbitrary units.

60°C, with increments of 1°C for 15 seconds. Data analysis was carried out with 7500 Software (Applied Biosystems). The auto threshold and baseline options were used for the calculations of Ct values per well. The linear equation for the standard curve (i.e., for preparations containing known quantities of DNA) was then used to interpolate the numbers of copies present in unknown samples. All samples showed only one Melting Temperature for the three targets, no primer-dimer formation was detected. qPCR reactions used primer sets designed from sequences conserved in both rodent and human species.

Statistical analysis

Probability values (*p* values) were computed using Student's *t*-test and expressed relative to sham group or saline-treated group. Group size for treatment groups was 6 animals except for the sham group in the CLP model where n=4.

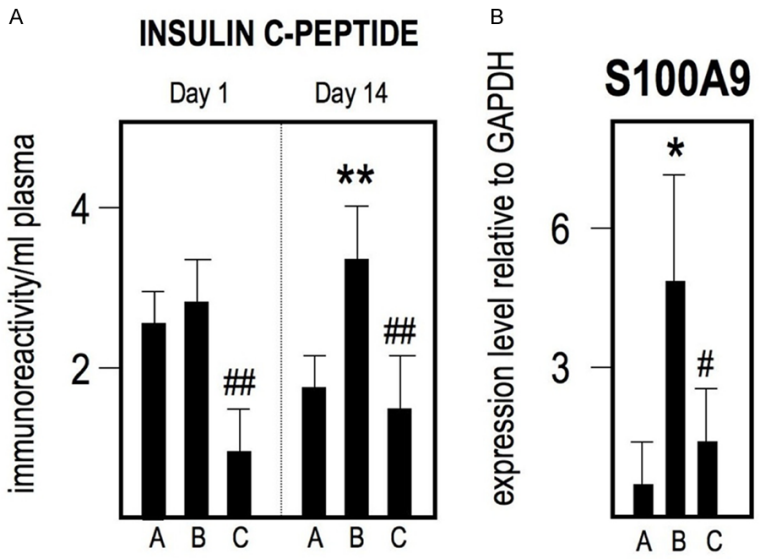

Figure 3. Rat scald model: plasma insulin C-peptide and kidney S100A9 gene expression. A=sham group (n=6); B=burn+saline group (n=6); C=burn+nephrilin group (n=6); Left Panel: Plasma insulin C-peptide ELISA immunoreactivity (arbitrary units per ml plasma); Right Panel: qPCR analysis of S100A9 transcripts relative to GAPDH in kidney tissue; *p<0.05 **p<0.01 (group A versus B); #p<0.05 ##p<0.01 (group B versus group C).

Results

Scald model

Using a well-validated rat scald burn model [11], we injected rats daily either with saline or 4 mg/kg body weight nephrilin. Animal tissues were analysed at 24 hours post-burn (Day 1) and on Day 14, in order to capture acute and delayed phases of the NSR.

Figure 1A shows that nephrilin reverses significant acute elevations in plasma IL-6 and TNF-α caused by burn stress; a similar trend with IL-10 does not reach significance (p=0.22). IL-33 levels are significantly reduced by nephrilin treatment. A canonical feature of the NSR is the early release of SP from sensory nerves into tissues such as kidney. **Figure 1B** (top panel) shows that SP levels in kidney tissue are elevated in burned rats during the acute phase. This elevation is reversed by nephrilin treatment.

One of the signature events of the acute phase NSR is the invasion of organ tissue by PMNs in the absence of a classical trigger such as bacterial infection. These PMNs are of a sub-class (PMN-II) that secretes CCL2/MCP-1, believed to be a key mediator of the immunosuppression that leads to sepsis. To obtain an estimate of PMN involvement we measured cathepsin G activity in kidney tissue extracts at 24 hours. **Table 2** shows that Cathepsin G is significantly elevated in the saline burn group and that nephrilin treatment reverses this elevation.

The NSR has been tentatively associated with the activation of protein kinase C-beta (PKC-β) and TRPV1 isoforms in the dorsal root ganglia. In association with the adaptor protein AKAP 150, PKC-β is believed to activate TRPV1 by serine phosphorylation leading to a cascade of neurogenic events that include release of bioactive peptides SP and CGRP, and activation of downstream pro- and anti-inflammatory pathways. **Figure 1C** shows that PKC-β-II and CGRP levels are significantly elevated in DRG in the delayed phase but not in the acute phase. Nephrilin reduces the elevation in the DRG levels of both markers. Plasma CCL2, an important marker of immunosuppressive M2Mphi activation is also elevated in the 14-day samples from the burn group. Treatment with nephrilin significantly reduces the elevation in plasma CCL2 (**Figure 1B**, bottom panel).

Phosphorylation of the adaptor protein p66shc is a central event in mitochodrial ROS generation. Elevation of p66shc-mediated mitochondrial ROS production may be related to insulin resistance during the hypermetabolic (chronic) phase of the NSR. The burn plus saline group shows significantly elevated S36 phosphorylation of p66shc in kidney tissue extracts (**Figure 2**, left panel). This elevation corresponds to nuclear and perinuclear staining for phospho-p66shc-S36 in tubular epithelia (**Figure 2**, right panel B). Nephrilin significantly reverses the elevation of phosphorylation of p66shc.

Plasma C-peptide levels are chronically elevated in burned patients and rodent models of burn trauma. **Figure 3**, left panel, shows elevation of plasma C-peptide in the 14-day sample from burned rats. Nephrilin treatment reverses this elevation.

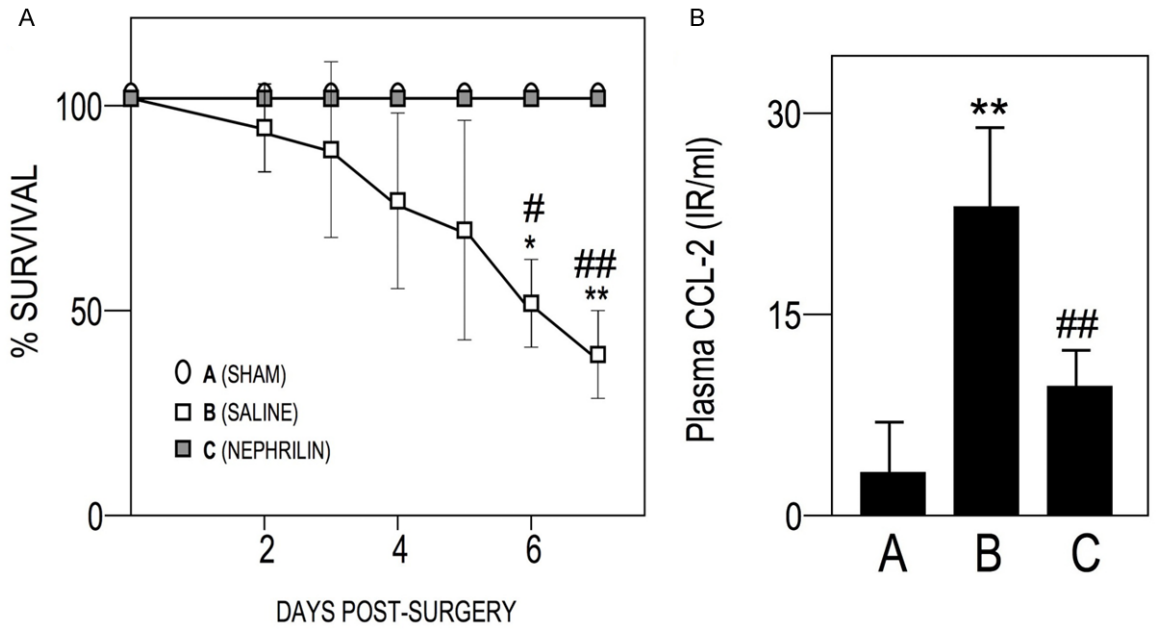

Figure 4. CLP model of sepsis. A=sham group (n=4); B=CLP+saline group (n=6); C=CLP+nephrilin group (n=6); Panel A: 3-day moving average percent survival (see methods; please note that error bars are not visible for groups A and C as all animals survived); Panel B: Plasma CCL2/MCP-1 ELISA immunoreactivity (arbitrary units/ml) at 24 hours post-surgery; AU=arbitrary units. *p<0.05 **p<0.01 (group A versus B); #p<0.05 ##p<0.01 (group B versus group C).

A previous study established that liver expression of the gene for S100A9/MRP14, a RAGE ligand associated with inflammation, insulin resistance and mortality, is significantly activated by scald injury in this rat model. In a preliminary test using gene arrays (data not shown) RNA extracted from bladder, kidney, lung and spleen also showed significant elevation (>5X) of S100A9 transcripts in the burn group but not in the burn plus nephrilin group. We therefore quantitated this elevation by qPCR using RNA extracted from kidney tissue. Nephrilin substantially reversed this elevation (**Figure 3**, right panel).

Cecal ligation and puncture model

The cecal ligation and puncture (CLP) mouse model of polymicrobial sepsis after surgical stress allows for quantitation of effects on short-term mortality - unlike the rat scald model, which has been optimized for mortality of <1%. Saline-treated mice exhibited approximately 70% mortality in 7 days. Nephrilin-treated animals were fully protected against mortality (**Figure 4A**). CCL2 levels at 24 hours were higher in the saline group. This elevation was reversed by nephrilin treatment (**Figure 4B**).

Discussion

In this work we have tested the effects of nephrilin peptide administration on the NSR using rodent models of burn trauma and surgical (cecal ligation) trauma. Each model is optimized for the recapitulation of different aspects of clinically important phenomena associated with burn trauma. The results of our study are consistent with the proposition that the dramatically higher incidence of sepsis observed in burn trauma (as opposed to other age-matched severe trauma) is suggestive of a mechanistic overlap in the neuroimmune response to burn and surgical traumas, and that treatment with nephrilin peptide may modulate the common underlying neuroimmune mechanism.

In the rat scald model, nephrilin reverses perturbations in the acute phase of the NSR: at 24 hours, elevations in pro-inflammatory markers (plasma IL-6 and TNF-α, tissue SP and S100A9 gene expression) are each reversed by nephrilin treatment. Perhaps because of high inter-animal variation, the dramatic rise in plasma IL-10 levels did not reach significance (p=0.24). IL-10 is believed to play an important role in

immunosuppression. Nephrilin appears to intervene at a point upstream of neutrophil infiltration, as levels of cathepsin G in kidney tissue are significantly lowered by nephrilin treatment. At a later time point (14 days), the burn-induced elevation of markers believed to be associated with immunosuppression, hypermetabolism and oxidative damage (plasma CCL2/MCP-1 and C-peptide, DRG levels of PKC-β-II and CGRP, and phosphorylated p66shc-S36 in kidney tissue) are also reversed by nephrilin. Interestingly, immunohistochemistry of kidney tissue slices using anti-p66shc-S36 antibody shows punctate staining in the nuclear and perinuclear region of tubular epithelial cells, which are known to be particularly sensitive to oxidative stress. In a previous study very similar punctate staining was observed as a result of hypertensive stress [7]. The exact sequence of mitochondrial ROS and inflammation in traumatic stress is not well understood. We demonstrate that, at least in this model of trauma, significant elevation of inflammatory markers such as IL-6 in plasma precedes the chronic elevation in mitochondrial ROS generation in kidney tissue. We do not know if a similar temporal sequence is to be found in other tissues or models.

In the mouse CLP model nearly 70% mortality from sepsis is observed in the control group, but treatment with nephrilin completely abolished mortality during the 7-day duration of the experiment. Furthermore, when compared to sham, there were significant elevations in plasma CCL2/MCP-1 for the CLP+saline group. Nephrilin treatment reversed this observed elevation in plasma CCL2/MCP1.

The current study has a number of limitations. We have demonstrated the protective effect of nephrilin administered concurrent with stress, but it would be useful to ask whether the same effect might be observed if treatment with nephrilin were initiated at a later time e.g. some hours or days after the initial burn or surgical stress. This is an area for future study. One other possible area for future investigation may involve additional established models of burn trauma and sepsis.

mTORC2 complex is an evolutionarily conserved kinase whose pleitropic functionality in mammalian cells has been implicated in cytoskeletal changes initiated in response to environmental conditions. Mechanistically, mTORC2 kinase appears to act via phosphorylation of AGC family kinases such as PKC, SGK and Akt. In our previous work we have reported that nephrilin, designed as a specific cell-penetrating inhibitor of mTORC2, reversed cellular responses to xenobiotic and metabolic stress when administered subcutaneously to whole animals [6, 7]. In this work we demonstrated the effect of nephrilin treatment in reversing the elevation of markers of neuroimmune response to traumatic stress in two rodent models. These observations, taken together, suggest the possibility that mTORC2 may play a role in the clinically important neuroimmune stress responses to trauma. The use of selective inhibitors of mTORC2 may represent an important new avenue for intervention in sepsis and burns.

Burn patients are three times as likely to succumb to sepsis as other age-matched trauma patients and over 200,000 patients die of sepsis in the ICU/CCU each year. Given the clinical importance of sepsis in critical care medicine generally, and in burn patients in particular, further investigation of the possible use of nephrilin in controlling the NSR in burn patients seems warranted.

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Disclosure of conflict of interest

None.

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