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The matri-cellular proteins 'cysteine-rich, angiogenic-inducer, 61' and 'connective tissue growth factor' are regulated in experimentally-induced sepsis with multiple organ dysfunction

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Abstract

Organ failure is a severe complication in sepsis for which the pathophysiology remains incompletely understood. Recently, the matri-cellular cysteine-rich, angiogenic induced, 61 (*Cyr*61/CCN1); connective tissue growth factor (*Ctgf*/CCN2); and nephroblastoma overexpressed gene (*Nov*/CCN3) (CCN)-protein family have been attributed organ-protective properties. Their expression is sensitive to mediators of sepsis pathophysiology but a potential role in sepsis remains elusive. To provide an initial assessment, 50 rats were subjected to 18 h of cecal-ligation and puncture or sham operation. Hepatic and pulmonary *CCN1* mRNA displayed an average 7.4- and 3.3-fold induction, while its cardiac expression was unchanged. The changes coincided with excessive hepatic and pulmonary inflammatory gene activation and a restricted cardiac inflammation. Furthermore, hepatocytes displayed a dosage-dependent *CCN1* mRNA response *in vitro*, supporting a cytokine-mediated CCN1 regulation in sepsis. *CCN2* mRNA was 2.2-fold induced in the liver, while 2.0-fold and 1.4-fold repressed in the heart and lung. Meanwhile, it did not respond to TNF-α exposure *in vitro*, which indicates different means of regulation than for CCN1. Taken together, this study provides the first evidence for multi-organ regulation of CCN1 and CCN2 in early stages of sepsis, and implies the eruption of inflammatory mediators as a potential mechanism behind the observed CCN1 regulation.

Keywords

Connective tissue growth factor, cysteine-rich, angiogenic inducer, 61, multiple organ failure, nephroblastoma overexpressed gene, sepsis

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Introduction

Sepsis is a complex disease that results from a harmful host response to severe infection.[1] Despite improvements in our understanding of its pathophysiology, mortality remains high. The presence of concurrent organ failure, in particular, is a strong predictor of sepsis-related death.[2] The pathophysiologic mechanisms behind sepsis-induced multiple organ failure (MOF) are, however, still controversial. Current knowledge implies a role of microvascular, and mitochondrial dysfunction, as well as leukocyte-mediated parenchyma disturbances.[3–7]

The extra cellular matrix (ECM) has received little attention in sepsis research. However, the identification and investigation of a new family of matri-cellular proteins, termed the CCN-family, suggests a potential role in sepsis-induced MOF.[8,9] The CCN-family is the acronym derived for the first three family members: cysteine-rich, angiogenic inducer, 61 (Cyr61/CCN1); connective tissue growth factor (Ctgf/CCN2); and nephroblastoma overexpressed gene (Nov/CCN3). The family was later expanded to include three

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additional members (WNT1 inducible signaling pathway protein 1-3; Wisp1-3/CCN4-6); the proteins are now denoted CCN1-CCN6.[8]

The biologic functions of the CCNs contrast with those of classical ECM proteins, as they serve to modulate cellular responses to environmental disturbances rather than sustaining structural roles.[8,9] Functioning as autocrine and paracrine multi-functional signal integrators, they support the adhesion, migration and proliferation of endothelial cells, as well as the proliferation and adhesion of vascular smooth muscle cells *in vitro* and drive angiogenesis *in vivo*.[10–16] The proteins play a potential role also on the endothelial surface, as they act as adhesion substrates for activated platelets and macrophages.[17,18] Moreover, they regulate macrophage migration and pro-inflammatory signaling through receptors widely distributed on innate immune cells and promote cytokine and chemokine expression.[19,20] Lastly, the CCNs engage in regulation of reactive oxygen species (ROS) accumulation, thereby affecting another pivotal element of inflammation and potentially interfering with vascular tone control.[21–25]

Furthermore, the expression of the CCN proteins is highly susceptible to cytokine and growth factor expression, hypoxia, hyperoxia, ROS and infections.[23] These features are essential parts of the septic environment, as well as involved in the pathophysiologic background of endothelial, mitochondrial and leukocyte dysfunction which is observed in sepsis-induced MOF.[3–5,8,26,27] This provides compelling evidence that the CCN expression might be affected in sepsis and, hence, hints to the potential involvement of CCNs in sepsis and MOF pathophysiology. In fact, renal CCN1 induction has been documented in a mouse model of poly-microbial sepsis.[28] However, the study presented here is the first multi-organ investigation of CCN expression in experimentally-induced sepsis *in vivo*.

Materials and methods

Animal model

Fifty male Wistar rats (Taconic, Hudson, NY, USA), weighing between 250 and 300 g, were kept under a 12/12 h light/dark cycle with unlimited access to food and water. The animals were randomized to cecal-ligation and puncture (CLP) or sham operation as previously described.[29] Briefly, the cecum was assessed through a midline laparotomy, ligated with 3-0 silk suture and punctured in two places with an 18-G needle. With this injury, approximately 10% mortality was observed within 18 h. The animals received saline (3 ml/100 g) and analgesia (Temgesic 0.05 mg/kg) subcutaneously following surgery, as well as 9 h postoperative. Eighteen hours after surgery a re-laparotomy was preformed and the animals were sacrificed by terminal bleeding from the abdominal aorta, using a 23-G needle and 10-ml syringe. Blood was collected for biochemical analysis and organs (lungs, livers and hearts) were harvested. All animal experiments were approved by the local animal care committee and conducted in accordance with the national animal welfare guidelines.

Biochemical markers

Levels of alanine transaminase, aspartate transaminase and troponin-T were measured in heparin anti-coagulated blood by the Department of Clinical Biochemistry (Oslo University Hospital, Rikshospitalet, Norway), using validated assays for routine patient analysis. Total leukocyte and platelet counts were done on EDTA anti-coagulated blood with assays validated for routine patient analysis. Arterial blood gas analysis was performed on heparin anti-coagulated blood in capillary tubes using an ABL 800 Flex analyzer (Radiometer Medical Aps, Copenhagen, Denmark).

Gene expression analysis

Total RNA was isolated from snap-frozen tissues by means of RNeasy mini kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. Samples were treated with an RNase free DNase set (Qiagen) to remove genomic contamination. RNA quality was controlled at random using the Experion™ automated electrophoresis system (BioRad Laboratories Inc., Hercules, CA, USA). Reverse transcription was done using a TaqMan RT kit (Applied Biosystems, Foster City, CA, USA).

RT-PCR was run in triplicates using a relative standard curve approach. *CCN1-CCN6 and RN18S1* (ribosomal protein S18, internal control) were analyzed using pre-inventoried TaqMan assays according to the manufacturer's instructions (Applied Biosystems). Cytokine and chemokine expressions were analyzed by SYBR Green technology, using 2× Universal master mix (Applied Biosystems), cDNA template, and 300 nM sense and anti-sense primers. SYBR Green primers were designed to span intron junctions. Specificity of the SYBR Green primers was assessed by melting point analysis. All assays were optimized to CT-value between 20 and 30 cycles. The primers utilized to amplify target genes were: *TNF-α*, forward 5′- ATG GCC CAG ACC CTC ACA CTC A, reverse 5′- CCG CTT GGT GGT TTG CTA CGC; *IL-1β*, forward 5′- GAC CTG TTC TTT GAG GCT GAC A, reverse 5′- CTC ATC TGG ACA GCC CAA GTC; *IL-6*, forward 5′- TAG TCC TTC CTA CCC CAA CTT C, reverse 5′- TTG GTC CTT AGC CAC TCC TTC; *CXCL1* forward 5′- CAA TGA GCT GCG CTG TCA GT, reverse 5′- TTG AAG TGA ATC CCT GCC ACT; *IL-10* forward 5′- AGC TGC GAC GCT GTC

ATC GAT, reverse 5′- CAC CTG CTC CAC TGC CTT GCT T.

Western blotting

Snap-frozen tissue samples were gently homogenized in RIPA buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific Inc., Waltham, MA, USA) prior to 30 min incubation on ice. The lysates were centrifuged twice at 10000 *g* for 10 min to remove insoluble material and the total protein concentration was determined by Bradford assay (BioRad). Proteins from randomly selected animals, with mRNA expression close to the average mean, were separated by 10% SDS-PAGE and transferred to a Hybond-N membrane (Amersham Bioscience, Amersham, UK). Blocking was carried out by incubating membranes with 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 1 h at room temperature (20–22°C). Membranes were incubated with primary Abs against mouse-CCN1, mouse-CCN3 (R&D systems, McKinley Place NE, MN, USA), human-CCN2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Cell Signaling technology Inc., Beverly, MA, USA) 18–20 h at 4°C. After washing three times with TBST, the membranes were incubated with a secondary anti-sheep, anti-goat or anti-rabbit Ab (Santa Cruz) for 1 h at room temperature and proteins visualized by LumiGlo (KPL, Inc., Gaithersburg, MD, USA). Displayed images were developed using a Kodak Image station 4000 mm Pro (Kodak, New Haven, CT, USA).

Immunohistochemistry

Paraffin-embedded pulmonary sections (6 μm) were rehydrated and blocked 1 h prior to incubation with purified anti-CCN2, raised as previously described.[30] Subsequently, CCN2 staining was performed with an avidin-biotin-peroxidase system, Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. Visualization was done by 3,3′-diaminobenzine (DAP)-staining (Thermo Fisher) and a hematoxylin counter-stain.

Cell culture

The human hepatocyte carcinoma cell line HepG2 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were maintained in EMEM (M2279) (Sigma Aldrich, St. Louis, MO, USA) with 10% FCS, L-glutamine, non-essential amino acids, and penicillin/streptomycin in 5% CO_2 at 37°C. Cells were sub-cultured twice weekly. For experiments, the cells were seeded in six-well plates at a density of 1.75 million/cells per ml, 2 ml per well, 24 h prior to stimulation with recombinant TNF-α (R&D systems).

Statistical analysis

Data distribution was evaluated in inverse normal plots and by means of the Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally-distributed data were analyzed by one-way ANOVA with Bonferroni or Thamhane's T2 post-test or by Students *t*-test. When normal distributions were violated, analyses were done by Mann-Whitney U test or Kruskal-Wallis multiple comparison test, followed by Mann-Whitney U post-test with Bonferroni adjustment. Differences were considered significant at the level of $P < 0.05$.

Results

CLP animals suffer from early sepsis with inflammatory organ impairment

The CLP animals presented with leukopenia, relative thrombocytopenia and hypoglycemia, while serum lactate levels were unaffected (Table 1).

Hepatic alanine transaminase and aspartate transaminase were significantly elevated, consistent with inflammatory liver injury. Severely elevated troponines is indicative of sepsis-induced cardiac failure;[31] however, this marker displayed only a minor increase among the CLP animals. The blood gas analysis revealed an unchanged pH value and $PaCo_2$ among the CLP animals, while PaO_2 and, hence, the PaO_2/FIO_2 ratio were significantly decreased (Table 1). To further address this pulmonary inflammatory impairment, selected tissue sections were evaluated by means of light microscopy. No visible signs of inflammatory cell infiltration or histological damage were, however, observed (Figure 1).

Table 1. Animal characteristics.

	Sham	CLP
Leukocytes, $\times 10^6$/ml	5.0 ± 0.3	1.0 ± 0.1[c]
Platelets, $\times 10^6$/ml	835 ± 26	645 ± 36[c]
Lactate, mM	2.2 ± 0.1	2.7 ± 0.4
Glucose, mM	10.5 ± 0.2	7.0 ± 0.3[c]
ALT, U/L	63.6 ± 2.3	178.7 ± 8.5[c]
AST, U/L	179.7 ± 9.4	381.0 ± 15.7[c]
$PaCo_2$, kPa	6.1 ± 0.2	5.7 ± 0.2
PaO_2, kPa	62.3 ± 0.9	56.4 ± 1.7[b]
PaO_2/FIO_2	296.8 ± 4.2	268.5 ± 8.0[b]
pH	7.42 ± 0.01	7.42 ± 0.02
TnT, ng/l	12.9 ± 0.8	22.9 ± 4.3[a]

Serum markers of inflammation and organ affection deviate in the CLP animals at 18 h. Values expressed as mean ± SEM. ALT: alanine transaminase, AST: aspartate transaminase, TnT: troponin-T. Statistical analysis was done by student's *t*-test. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$.

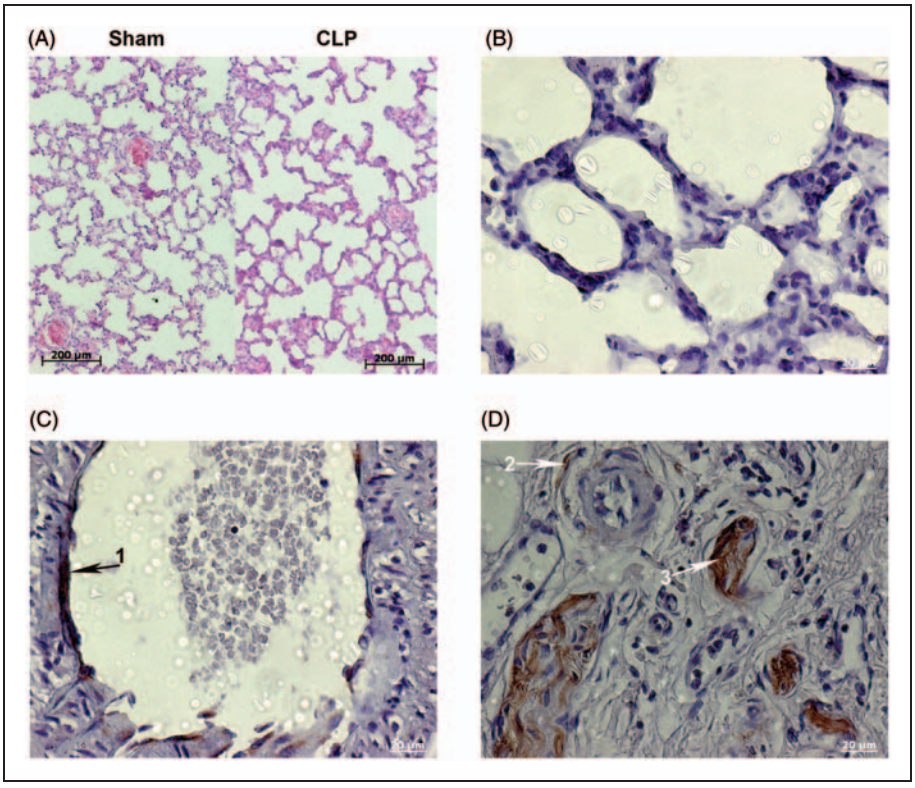

Figure 1. Lung morphology and CCN2 protein expression. Hematoxylin and eosin stained lung sections from cecal ligation and puncture (CLP) and sham operated animals at 18 h (A). CCN2 immunohistochemistry (B–D) were done to identify involved cell types. The alveolar septa (B) showed no protein expression, while intense signals were observed in the endothelial lining of the larger vessels (C, arrow 1), and in the extra cellular matrix (D, arrow 3), as well as around the minor airways (D, arrow 2) in the central lung parts.

CLP animals display tissue-specific patterns of inflammation

To evaluate the inflammatory insult on the organ parenchyma hepatic, pulmonary and cardiac homogenates were analyzed for *TNF-α*, *IL-1β*, *IL-6*, chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (*CXCL1,* an analog to human *IL-8*), and *IL-10* mRNA expression (Table 2).

Average expression of cardiac and hepatic pro-inflammatory genes revealed similar patterns. In both tissues, *IL-1β* was the most induced gene, while the *TNF-α* and *IL-6* increases were alike. Meanwhile, average hepatic induction of these genes was excessive and cardiac expression only limited, clearly showing differences in the extent of pro-inflammatory activation. Indicative of differences in the pro-inflammatory process, the average pulmonary mRNA expression manifested a vastly induced *IL-6*, followed by medium *TNF-α*, and minimum *IL-1β* expression.

Peak expression of *CXCL1* was observed in the heart, closely followed by lungs, while being less in the liver. Furthermore, pulmonary *IL-10* expression was highly induced, while hepatic expression was restricted and cardiac expression non-detectable. This picture clearly demonstrates tissue specific patterns of inflammatory activation.

The CLP animals divide in high and low inflammation responders

Based on the inflammatory gene activation, the CLP animals were divided into two sub-groups (division criterion was a *TNF-α* fold-induction of <5 or >8). One, termed 'high inflammation' (HI), presented with excessive mRNA induction while the other, termed 'low inflammation' (LI) had only modest gene activation. This division was evident for all hepatic and pulmonary inflammatory genes tested, except for pulmonary *IL-10*, but not observed for any of the genes in the heart (Table 2).

There was no correlation between the HI/LI groups and changes in measured markers of organ function or hematologic parameters (data not shown).

CCNs are regulated in early stages of sepsis-induced organ impairment

To initially investigate CCN regulation in sepsis, *CCN1-CCN6* mRNA expression levels were screened in a small pilot experiment utilizing 10 rats (5 CLP and 5 sham; Table 3). Based on this experiment, CCN1–CCN3 were selected for further investigations.

Analysis of hepatic *CCN1-CCN3* mRNA expression (Figure 2A–C) revealed an average 7.4-fold *CCN1*,

Table 2. Inflammatory gene expression.

Hepatic	CLP average	LI	HI
TNF-α	14.3[c]	2.8[c]	32.7[e]
IL-1β	25.7[c]	7.1[c]	55.3[e]
IL-6	11.8[a]	0.7	29.4[e]
CXCL1	10.7[c]	5.8[c]	18.5[e]
IL-10	4.6[c]	2.3[c]	8.1[e]
Pulmonary	CLP average	LI	HI
TNF-α	10.9[c]	8.2[c]	15.2[d]
IL-1β	5.0[c]	2.5[c]	8.9[e]
IL-6	66.9[c]	6.1[c]	164.0[d]
CXCL1	21.4[c]	12.8[c]	35.1[d]
IL-10	57.4[c]	55.3[c]	67.5
Cardiac	CLP average	LI	HI
TNF-α	3.4[c]	3.5[c]	3.4
IL-1β	11.3[c]	11.4[c]	11.3
IL-6	5.5[b]	4.1	7.7
CXCL1	24.9[c]	18.5[c]	36.4
IL-10	ND	ND	ND

Hepatic, pulmonary and cardiac inflammatory gene expressions. Values expressed as fold induction of sham mean. Statistical analysis of two groups were performed by Mann-Whitney U test and for three groups by Kruskal-Wallis test followed by Mann-Whitney U post-test with Bonferroni adjustment. CLP average: Fold change in the collective cecal ligation and puncture group. LI: fold change in the LI group. HI: fold change in the HI group. *CXCL1*: chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha). [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ against sham, [d]$P < 0.05$ and [e]$P < 0.001$ against LI. ND: not detectable with the utilized assay.

Table 3. *CCN1-CCN6* mRNA expressions in early sepsis.

	CCN1	*CCN2*	*CCN3*	*CCN4*	*CCN5*	*CCN6*
Hepatic	DT	DT	DT	ND	ND	ND
Pulmonary	DT	DT	DT	ND	DT	ND
Cardiac	DT	DT	DT	DL	DL	ND
Renal	DT	DT	DT	DT	DT	ND
Spleen	DT	DT	DT	ND	ND	ND

Pilot experiment investigating *CCN1-CCN6* mRNA regulation in the liver, lung, heart, kidney and spleen of rats subjected to cecal ligation and puncture (CLP) ($n = 5$) or sham operation ($n = 5$) for 18 h. DT: detectable (CT < 30) in both CLP and sham group, DL: Detectable (CT < 30) in one of the groups, ND: not detectable (CT > 30) in either group.

a 2.1-fold *CCN2* and a 2.4-fold *CCN3* mRNA induction in CLP animals at 18 h.

Interestingly, the LI/HI groups defined by inflammatory activation were also uncovered for *CCN1* mRNA expression, which was not reflected in *CCN2* or *CCN3* mRNA patterns. The *CCN1* mRNA expression in the HI group exhibited a 17-fold induction while the mRNA expression in the LI group remained at the level of sham. Statistical analysis confirmed strong correlations between *CCN1* mRNA expression and the gene expression of *TNF-α* (0.889, $P < 0.01$) (Figure 2D), *IL-1β* (0.811, $P < 0.01$), *IL-6* (0.813, $P < 0.01$), *CXCL1* (0.711, $P < 0.01$), and *IL-10* (0.868, $P < 0.01$). Contradicting this, *CCN2* mRNA presented with weak correlations to *TNF-α* (0.397, $P < 0.05$) and *IL-10* (0.400, $P < 0.05$) only, and *CCN3* mRNA with *IL-6* (0.424, $P < 0.05$).

Western-blot analysis confirmed CCN2 protein regulation, but failed to detect changes in CCN1 and CCN3 protein levels (data not shown).

Pulmonary *CCN* expressions (Figure 3A–C) revealed an average 3.3-fold *CCN1* mRNA induction, a twofold *CCN2* mRNA reduction and a 2.2-fold *CCN3* mRNA increase. The HI/LI distribution was also maintained for *CCN1* mRNA expression in this tissue. The HI group exhibited a 4.8-fold mRNA induction and, in contrast to the hepatic situation, the LI group exhibited a 2.3-fold mRNA induction. As for the liver, neither *CCN2* nor *CCN3* mRNA expression reflected the HI/LI pattern. Meanwhile, evident correlations between *CCN1* mRNA and gene expression of tested inflammatory markers were only observed for *IL-1β* (0.537, $P < 0.01$) and *IL-6* (0.594, $P < 0.01$) in this tissue. In contrast to hepatic *CCN2* mRNA induction, the pulmonary *CCN2* gene was repressed, manifesting tissue-specific *CCN2* mRNA responses (Figures 2B and 3B).

Pulmonary CCN1 and CCN2 protein regulation was confirmed by means of Western blot, while CCN3 protein was unchanged (Figure 3D). To identify the potentially involved and affected cell types, CCN2 protein expression was evaluated by immunohistochemistry (Figure 1B–D). Staining was seen around the minor bronchia (arrow 2), in the ECM (arrow 3) and the endothelial lining of larger vessels (arrow 1) in the central lung parts. Furthermore, protein expression was observed in vascular and airway smooth muscle cells (not shown). In contrast, no staining was observed in the distal alveolar part of the lungs (Figure 1B).

Cardiac *CCN1* and *CCN3* expression were unchanged, while *CCN2* gene expression was repressed by 30% ($P < 0.05$, data not shown).

Recombinant TNF-α regulates hepatocyte CCN1 but not CCN2 or CCN3 mRNA levels

With the aim to further address the molecular mechanisms behind the observed hepatic *CCN1–CCN3* mRNA regulation, and to provide evidence for *CCN* regulation in non-immune cells, as well as investigating its relevance in a human cell-type, *in vitro* studies were conducted in HepG2 cells (Figure 4). The cells were stimulated with increasing dosages of recombinant TNF-α for 24 h and analyzed for *CCN1-CCN3* mRNA expression.

CCN1 mRNA expression (Figure 4A) showed a clear, dose-dependent response while *CCN2* mRNA expression (Figure 4B) was unaffected. *CCN3* persisted below the detection limit of the assay system used

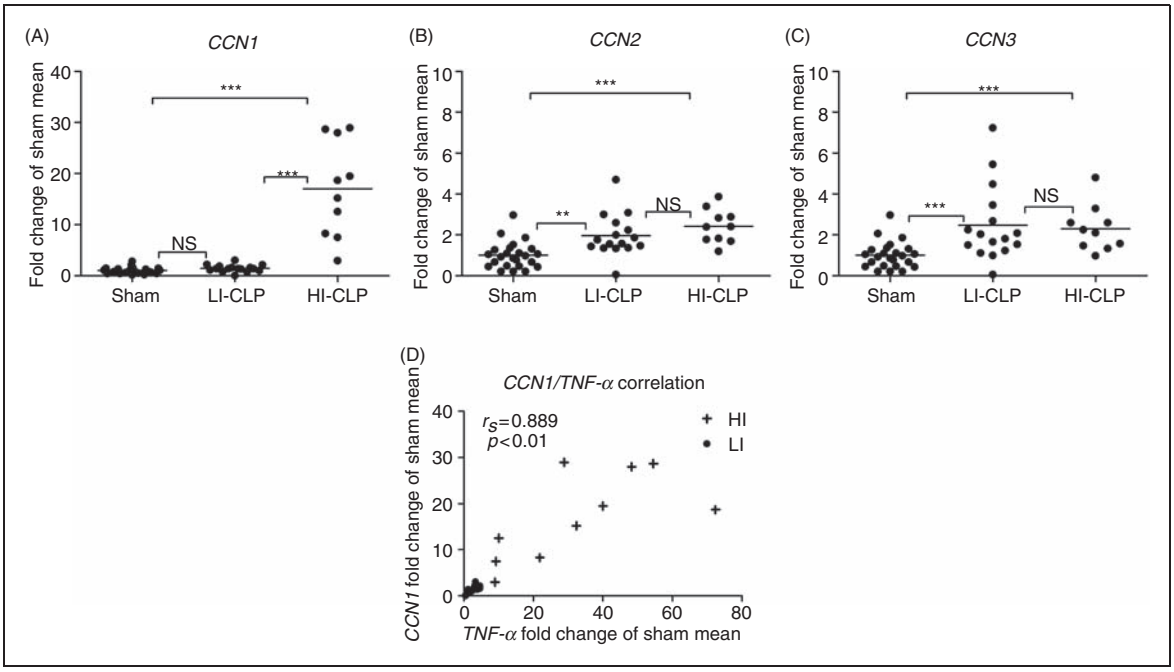

Figure 2. Hepatic *CCN1*, *CCN2*, and *CCN3* mRNA expressions are induced in early sepsis. Gene expressions were analyzed in whole liver homogenates by means of RT-PCR and are expressed as fold induction of sham mean. Individual animals represented by dots amid, average as horizontal lines (A) *CCN1*, (B) *CCN2,* and (C) *CCN3*. LI-CLP: low Inflammation- cecal ligation and puncture (CLP) group, HI-CLP: high Inflammation-CLP group. Correlation of *CCN1* and *TNF-α* mRNA expressions in the CLP animals (D). Animals in the LI group (•) scatter in the lower left corner, while HI animals (+) spread towards the upper right. Statistics were done by Kruskal-Wallis test, followed by Mann-Whitney U post-test with Bonferroni correction. Correlations (D) were calculated by Spearman rho. Results were considered significant at alpha level (or adjusted alpha level) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

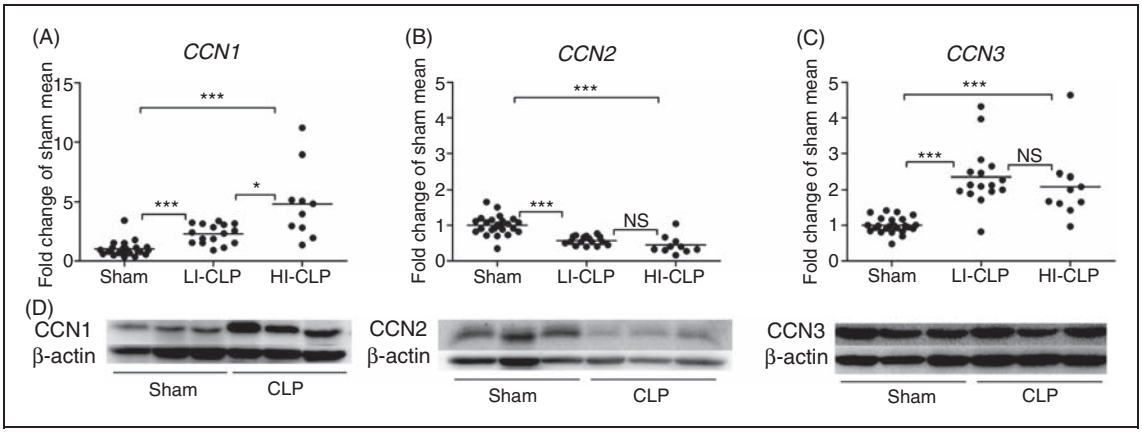

Figure 3. Pulmonary *CCN1*, *CCN2* and *CCN3* expressions. *CCN1* (A), *CCN2* (B) and *CCN3* (C) gene expressions were analyzed by means of RT-PCR and are expressed as fold change of sham mean. Individual animals represented by dots amid average as horizontal lines. LI-CLP and HI-CLP indicates LI and HI cecal ligation and puncture (CLP) animals. Regulation on the protein level was confirmed by Western-blot (D) in randomly selected animals, with gene expression at the level of the average mean. CCN1-specific bands corresponding to the glycosylated full-length CCN1 (50 ku) and a un-glycosylated form at 40 ku was observed. Regulation was detected at the 40 ku band as shown. Statistical analyses were done by Kruskal-Wallis test, followed by Mann-Whitney U post-test with Bonferroni correction. Results were considered significant at the alpha level (or adjusted alpha level): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

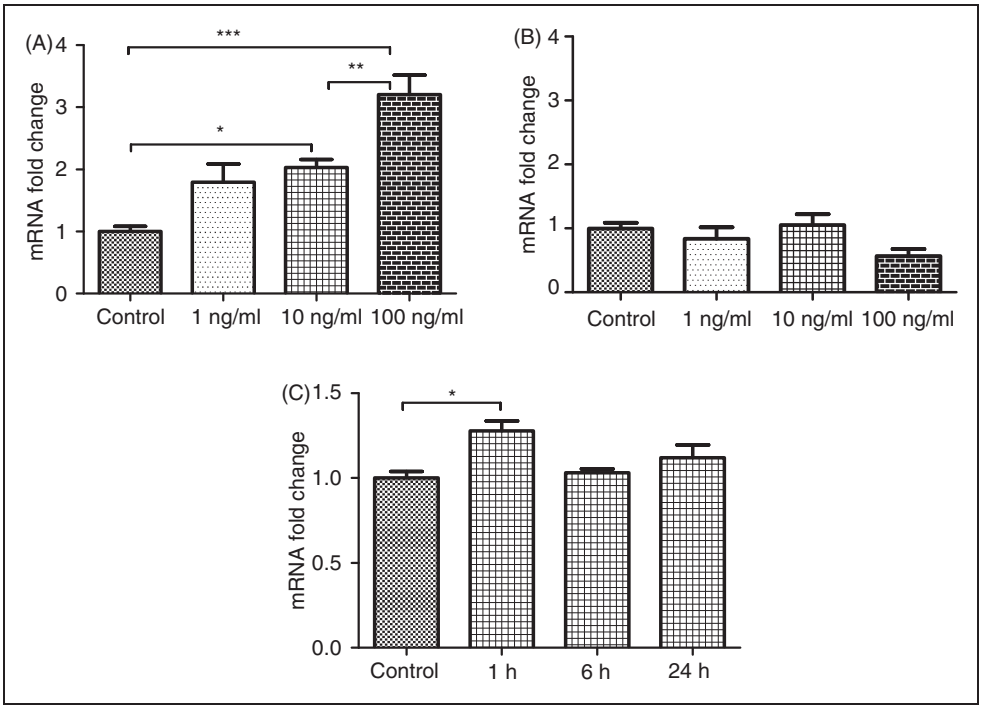

Figure 4. Hepatocyte *CCN1* expression responds to TNF-α exposure. HepG2 cells were stimulated 24 h with increasing dosages of TNF-α (1, 10 and 100 ng/ml) and *CCN1* (A), and *CCN2* (B) gene expression profiles were analyzed by means of RT-PCR. A pulse-chase experiment was conducted to confirm the specificity of *CCN1* induction by TNF-α. The cells were stimulated for 1 h with TNF-α and *CCN1* expression tested after 1, 6 and 24 h. Data are obtained from three independent experiments conducted in triplicate. Values standardized to control mean and expressed as mean ± SEM. Statistical tests were performed by one-way ANOVA with Bonferroni post-test. Results were considered significant at the level of: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(not shown). To investigate the potential unspecific effects of TNF-α stimulation on cell metabolism a pulse-chase experiment was conducted (Figure 4C). The cells were stimulated with 10 ng of TNF-α for 1 h and *CCN1* mRNA expression was analyzed consecutively for 24 h. At 1 h, CCN1 was significantly induced while returning to baseline after 6 h.

Discussion

The present study provides novel evidence for simultaneous regulation of the matri-cellular CCNs in different organs as a response to sepsis-induced impairment. It provides clear evidence for a connection between CCN1 regulation and the expression of both pro-inflammatory and anti-inflammatory cytokines, as well as chemokines. The data indicate that this connection is mediated through cytokines and chemokines provoking CCN1 responses. The experiments also demonstrate CCN2 to be regulated in a tissue-specific manner that is adverse in some organs. Furthermore, it also implies that CCN3 is regulated. The underlying mechanisms of CCN2 and CCN3 regulation seem, however, more complicated than for CCN1. Lastly, our pilot study identified all members of the CCN-family, except for CCN6, as potentially affected in the early stages of sepsis-induced organ impairment.

The gene expression profiles of cytokines and chemokines were analyzed to delineate the environment in which we studied CCN expression. In accordance with the notion of a compartmentalized inflammatory response in sepsis,[32,33] tissue-specific profiles were observed in the three organs. However, isolated evaluation of the pro-inflammatory genes revealed hepatic and cardiac profiles to be similar, though different in magnitude, while the pulmonary profile was disparate. Similar observations have been made also by others[34,35] and could indicate pulmonary inflammation to be different from the inflammation in other organs. Moreover, the septic rats divided into LI and HI responders, which is likely to reflect different stages of disease progression or inter-individual differences in susceptibility to the infection.

Interestingly, these inflammatory-based LI and HI groups were also seen in hepatic and pulmonary *CCN1* mRNA expression. Simultaneously, *CCN1* mRNA regulation was not detected in the heart which exhibited limited inflammation. Furthermore, the mRNA levels correlated strongly to tested inflammatory markers in the liver. These observations provide strong evidence for a biological dependency between CCN1 and multiple cyto- and chemokines *in vivo*. In the lungs, however, the correlations were evident only for *IL-6* and *IL-1β*. However, this may well be

explained by a relatively small difference between *CCN1* mRNA induction in the pulmonary LI and HI groups. Conversely, the hepatic LI group elicited *CCN1* expression at the level of sham thereby presenting a more substantial difference between the LI and HI groups. That *CCN1* mRNA was induced in the lung LI group but not in the liver LI group, was likely caused by the distinct nature of the inflammatory response or tissue differences of *CCN1* susceptibility.

The causal connection between *CCN1* and cytokine/chemokine expressions, as well as the cellular source of the CCN1 responses, remains to be identified. Human peripheral blood monocytes demonstrate increased *CCN1* mRNA expression after challenge with endotoxin *in vitro*.[36] This, in conjunction with *CCN1* obeying the LI/HI patterns and displaying close associations to inflammatory markers, could indicate the observed *CCN1* response to originate from inflammatory cell types. However, several lines of evidence also support expression of the autocrine and paracrine acting CCNs from other cells-types, both stromal and parenchymal ones.[8,9] Our studies clearly demonstrate a *CCN1* mRNA response in hepatocytes upon TNF-α exposure. This implies that the observed CCN1 regulation *in vivo* might partially emerge from parenchyma cells, and that the parenchymal CCN1 regulation can be a consequence of environmental cytokine/chemokine exposure. Hereby, the data implicate a role for CCN1 in the parenchymal response to sepsis.

The observed CCN2 regulation appears to be more complicated. The mRNA levels correlated with few inflammatory markers, the LI/HI division was absent and TNF-α did not elicit a *CCN2* mRNA response in the hepatocytes. This merely excludes cytokines and chemokines as main regulators of *CCN2* in the utilized model. Despite the elusiveness of regulatory factors, an interesting tissue-specific response was observed for CCN2. Such tissue-specific CCN2 responses have also been observed previously.[37,38] In these studies, TNF-α inhibited CCN2 expression in skin fibroblasts and conversely, induced CCN2 in mesangial cells *in vitro*. As such, the observed tissue-specific differences in CCN2 expression are likely to occur through similar mechanisms and this observation is encouraging further investigations.

The regulation of CCN1 and CCN2 on the protein level revealed a complex pattern. Our experiments uncovered CCN2 but not CCN1 protein regulation in the liver, while both proteins were regulated in the lungs. With a few exceptions, CCN proteins are regulated at the transcriptional level.[39] Moreover, *in vitro* studies have demonstrated that CCN2 responds more instantly to TNF-α stimulation than CCN1 does.[40] This indicates that the lack of CCN1 protein regulation in the liver is likely a result of a premature time point of analysis. The regulation of CCN1 in one organ and not in the other at 18 h could implicate tissue-specific differences in CCN1 sensitivity to environmental perturbations. Alternatively, it could be caused by tissue-specific differences in inflammatory profiles.

As the functional properties of the CCN proteins are diverse, current knowledge implicates potentially beneficial, as well as adverse, effects of these proteins in sepsis and MOF. CCN1 protects pulmonary epithelial cells from hyperoxia and nutrient deprivation which, in addition to its pro-angiogenic properties, implies a protective role in sepsis.[11,41] Furthermore, a recent study demonstrated cardio-protective properties of CCN1 over-expression in a mouse model of infectious myocarditis, promoting an organ-preserving role of CCN1.[42] CCN2 is also a pro-angiogenic factor, processing cardio-protective capabilities.[43] As such, the observed pulmonary and cardiac CCN2 suppression could be part of the vascular damage observed in sepsis.[3,5,24,44] Meanwhile, neonatal mice, over-expressing CCN2 in type-II epithelial cells, have recently been shown to suffer massive macrophage and neutrophil infiltration of the alveolar space.[45] Hence, the down-regulation of CCN2 may well represent a protective response.

In contrast to these potentially beneficial effects, the CCN proteins exert functions that may be detrimental in sepsis. The support of platelet adhesion to endothelial cells hints to involvement in intravascular clot formation and micro-vascular dysfunction.[17] Moreover, macrophages adhere to distinct CCNs on endothelial cells and some of the proteins induce macrophage pro-inflammatory signaling and chemotaxis.[18–20,46] Given the critical role of tissue infiltration with dysfunctional leukocytes, this promotes possible harmful effects of CCN proteins.[23,26,27]

The current study is the first multi-organ description of CCN proteins in sepsis-induced organ-impairment. It provides evidence for CCN1 regulation in early stages of sepsis and implies the eruption of cytokines/chemokines as a potential mechanism. Evidence is also presented for tissue-specific CCN2 regulation and for uniform *CCN3* mRNA regulation but seemingly through different mechanisms than for CCN1. Furthermore, the study indicates a potential regulation of CCN4 and CCN5 in sepsis. However, the diversity of CCN functions necessitates functional studies to assess the pathophysiologic impact of the presented data.

Conclusion

The current study is the first multi-organ investigation of CCNs in early sepsis. It provides evidence that CCN1 and CCN2 are regulated in rodents with CLP-induced sepsis. Furthermore, a close association between cytokine and *CCN1* gene expression was observed, and evidence was presented for TNF-α-induced *CCN1* mRNA expression *in vitro*. The study is encouraging for functional investigations

on the role of CCN proteins in sepsis and sepsis-induced multiple organ dysfunction.

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Conflict of interests

The authors declare that there are no conflicts of interest.

References

1. Bone RC, Balk RA, Cerra FB, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 1992; 101: 1644–1655.
2. Martin GS, Mannino DM, Eaton S, et al. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 2003; 348: 1546–1554.
3. Balestra GM, Legrand M and Ince C. Microcirculation and mitochondria in sepsis: getting out of breath. *Curr Opin Anaesthesiol* 2009; 22: 184–190.
4. Harrois A, Huet O and Duranteau J. Alterations of mitochondrial function in sepsis and critical illness. *Curr Opin Anaesthesiol* 2009; 22: 143–149.
5. Ince C. The microcirculation is the motor of sepsis. *Crit Care* 2005; 9: 13–19.
6. Peters K, Unger RE, Brunner J, et al. Molecular basis of endothelial dysfunction in sepsis. *Cardiovasc Res* 2003; 60: 49–57.
7. Brown KA, Brain SD, Pearson JD, et al. Neutrophils in development of multiple organ failure in sepsis. *Lancet* 2006; 368: 157–169.
8. Chen CC and Lau LF. Functions and mechanisms of action of CCN matricellular proteins. *Int J Biochem Cell Biol* 2009; 41: 771–783.
9. Kular L, Pakradouni J, Kitabgi P, et al. The CCN family: A new class of inflammation modulators? *Biochimie* 2011; 93: 377–388.
10. Babic AM, Chen CC and Lau LF. Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphavbeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. *Mol Cell Biol* 1999; 19: 2958–2966.
11. Babic AM, Kireeva ML, Kolesnikova TV, et al. CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. *Proc Natl Acad Sci USA* 1998; 95: 6355–6360.
12. Delmolino LM, Stearns NA and Castellot Jr JJ. COP-1, a member of the CCN family, is a heparin-induced growth arrest specific gene in vascular smooth muscle cells. *J Cell Physiol* 2001; 188: 45–55.
13. Lee HY, Chung JW, Youn SW, et al. Forkhead transcription factor FOXO3a is a negative regulator of angiogenic immediate early gene CYR61, leading to inhibition of vascular smooth muscle cell proliferation and neointimal hyperplasia. *Circ Res* 2007; 100: 372–380.
14. Leu SJ, Lam SC and Lau LF. Pro-angiogenic activities of CYR61 (CCN1) mediated through integrins alphavbeta3 and alpha6beta1 in human umbilical vein endothelial cells. *J Biol Chem* 2002; 277: 46248–46255.
15. Lin CG, Leu SJ, Chen N, et al. CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family. *J Biol Chem* 2003; 278: 24200–24208.
16. Shimoyama T, Hiraoka S, Takemoto M, et al. CCN3 inhibits neointimal hyperplasia through modulation of smooth muscle cell growth and migration. *Arterioscler Thromb Vasc Biol* 2010; 30: 675–682.
17. Jedsadayanmata A, Chen CC, Kireeva ML, et al. Activation-dependent adhesion of human platelets to Cyr61 and Fisp12/mouse connective tissue growth factor is mediated through integrin alpha(IIb)beta(3). *J Biol Chem* 1999; 274: 24321–24327.
18. Schober JM, Chen N, Grzeszkiewicz TM, et al. Identification of integrin alpha(M)beta(2) as an adhesion receptor on peripheral blood monocytes for Cyr61 (CCN1) and connective tissue growth factor (CCN2): immediate-early gene products expressed in atherosclerotic lesions. *Blood* 2002; 99: 4457–4465.
19. Bai T, Chen CC and Lau LF. Matricellular protein CCN1 activates a proinflammatory genetic program in murine macrophages. *J Immunol* 2010; 184: 3223–3232.
20. Cicha I, Yilmaz A, Klein M, et al. Connective tissue growth factor is overexpressed in complicated atherosclerotic plaques and induces mononuclear cell chemotaxis in vitro. *Arterioscler Thromb Vasc Biol* 2005; 25: 1008–1013.
21. Artigas A, Bernard GR, Carlet J, et al. The American-European Consensus Conference on ARDS, part 2: Ventilatory, pharmacologic, supportive therapy, study design strategies, and issues related to recovery and remodeling. Acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1998; 157: 1332–1347.
22. Chen CC, Young JL, Monzon RI, et al. Cytotoxicity of TNFalpha is regulated by integrin-mediated matrix signaling. *EMBO J* 2007; 26: 1257–1267.
23. Cohen J. The immunopathogenesis of sepsis. *Nature* 2002; 420: 885–891.
24. Matsuda N and Hattori Y. Vascular biology in sepsis: pathophysiological and therapeutic significance of vascular dysfunction. *J Smooth Muscle Res* 2007; 43: 117–137.
25. Miller DS and Sen M. Potential role of WISP3 (CCN6) in regulating the accumulation of reactive oxygen species. *Biochem Biophys Res Commun* 2007; 355: 156–161.
26. Abraham E and Singer M. Mechanisms of sepsis-induced organ dysfunction. *Crit Care Med* 2007; 35: 2408–2416.
27. Hotchkiss RS and Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med* 2003; 348: 138–150.
28. Miyaji T, Hu X, Yuen PS, et al. Ethyl pyruvate decreases sepsis-induced acute renal failure and multiple organ damage in aged mice. *Kidney Int* 2003; 64: 1620–1631.
29. Wang YY, Ryg U, Dahle MK, et al. Liver X receptor protects against liver injury in sepsis caused by rodent cecal ligation and puncture. *Surg Infect (Larchmt)* 2011; 12: 283–289.
30. Ahmed MS, Oie E, Vinge LE, et al. Connective tissue growth factor—a novel mediator of angiotensin II-stimulated cardiac fibroblast activation in heart failure in rats. *J Mol Cell Cardiol* 2004; 36: 393–404.
31. Maeder M, Fehr T, Rickli H, et al. Sepsis-associated myocardial dysfunction: diagnostic and prognostic impact of cardiac troponins and natriuretic peptides. *Chest* 2006; 129: 1349–1366.
32. Chinnaiyan AM, Huber-Lang M, Kumar-Sinha C, et al. Molecular signatures of sepsis: multiorgan gene expression profiles of systemic inflammation. *Am J Pathol* 2001; 159: 1199–1209.
33. Cavaillon JM and Annane D. Compartmentalization of the inflammatory response in sepsis and SIRS. *J Endotoxin Res* 2006; 12: 151–170.
34. Carlsen H, Moskaug JO, Fromm SH, et al. In vivo imaging of NF-kappa B activity. *J Immunol* 2002; 168: 1441–1446.
35. Yao L, Berman JW, Factor SM, et al. Correlation of histopathologic and bacteriologic changes with cytokine expression in an

experimental murine model of bacteremic Staphylococcus aureus infection. *Infect Immun* 1997; 65: 3889–3895.
36. Panutsopulos D, Arvanitis DL, Tsatsanis C, et al. Expression of heregulin in human coronary atherosclerotic lesions. *J Vasc Res* 2005; 42: 463–474.
37. Leask A and Abraham DJ. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *J Cell Sci* 2006; 119: 4803–4810.
38. Moritani NH, Kubota S, Sugahara T, et al. Comparable response of ccn1 with ccn2 genes upon arthritis: An in vitro evaluation with a human chondrocytic cell line stimulated by a set of cytokines. *Cell Commun Signal* 2005; 3: 6.
39. Abraham DJ, Shiwen X, Black CM, et al. Tumor necrosis factor alpha suppresses the induction of connective tissue growth factor by transforming growth factor-beta in normal and scleroderma fibroblasts. *J Biol Chem* 2000; 275: 15220–15225.
40. Cooker LA, Peterson D, Rambow J, et al. TNF-alpha, but not IFN-gamma, regulates CCN2 (CTGF), collagen type I, and proliferation in mesangial cells: possible roles in the progression of renal fibrosis. *Am J Physiol Renal Physiol* 2007; 293: 157–165.
41. Jin Y, Kim HP, Ifedigbo E, et al. Cyr61 protects against hyperoxia-induced cell death via Akt pathway in pulmonary epithelial cells. *Am J Respir Cell Mol Biol* 2005; 33: 297–302.
42. Rother M, Krohn S, Kania G, et al. Matricellular signaling molecule CCN1 attenuates experimental autoimmune myocarditis by acting as a novel immune cell migration modulator. *Circulation* 2010; 122: 2688–2698.
43. Ahmed MS, Gravning J, Martinov VN, et al. Mechanisms of novel cardioprotective functions of CCN2/CTGF in myocardial ischemia/reperfusion injury. *Am J Physiol Heart Circ Physiol* 2011; 300: 1291–1302.
44. Mutunga M, Fulton B, Bullock R, et al. Circulating endothelial cells in patients with septic shock. *Am J Respir Crit Care Med* 2001; 163: 195–200.
45. Chen S, Rong M, Platteau A, et al. CTGF disrupts alveolarization and induces pulmonary hypertension in neonatal mice: implication in the pathogenesis of severe bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol* 2011; 300: 330–340.
46. Sanchez-Lopez E, Rayego S, Rodrigues-Diez R, et al. CTGF promotes inflammatory cell infiltration of the renal interstitium by activating NF-kappaB. *J Am Soc Nephrol* 2009; 20: 1513–1526.