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Expression of CD44s and CD44v3 by Proximal Tubules Influences the Renal Inflammatory Milieu-Induced by LPS Injection

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Abstract

Systemic inflammatory response syndrome (SIRS) typically causes multiple-organ dysfunction/failure, including acute kidney injury (AKI). CD44 comprises a family of 85–200 kDa transmembrane glycoproteins that are widely expressed by multiple cell types and involved in a variety of inflammatory diseases. The multiple functions of CD44 have been attributed to the existence of numerous CD44 isoforms generated by alternative mRNA splicing as well as by extensive post-translational modifications. Renal CD44 expression is minimal in healthy adult kidneys, whereas in inflammatory renal disorders CD44 expression is markedly induced particularly in injured tubular epithelial cells (TEC) both in human diseases and in animal models. The function of CD44 on TEC remains unclear; previously we showed that the shortest isoform CD44 standard (CD44s) and the long CD44 variant 3-10 (CD44v3) exert opposite effects in fibrotic settings.

To assess the contribution of tubular expression of CD44s and CD44v3 in SIRS-associated AKI, we used WT and unique transgenic mice expressing CD44s or CD44v3 specifically on proximal TEC. Mice were subjected to intraperitoneal injection of LPS and were sacrificed 4 and 24 hours later. The presence of CD44-isoforms in TEC did not alter the onset of kidney dysfunction or lymphocyte influx but affected the induction of KIM-1 and pro-/anti-inflammatory cytokines after LPS injection. Transgenic kidneys expressing CD44s/CD44v3 displayed more KIM-1 expression, less TNF-α and IL-1β at 4 hours compared to WT kidneys. At 24 hours, CD44v3-expressing kidneys showed elevated IL-10 and TLR4 negative regulator mRNA levels.

Proximal TEC-CD44 influenced the renal inflammatory milieu and TEC-CD44v3 associated with expression of anti-inflammatory molecules.

Keywords: SIRS; Sepsis; AKI; CD44; CD44-variants; Tubules; Kidney; Inflammation; Cytokines; LPS

Introduction

Systemic inflammatory response syndrome (SIRS) typically causes multiorgan dysfunction and is frequently triggered by a harmful host response to primary infection (sepsis) [1,2]. Lipopolysaccharide (LPS) has been widely used in animal models to unravel pathological mechanisms of SIRS-related organ failure. Initially recognized by extra renal Toll-like receptor 4 (TLR4) [3], LPS triggers the production of cytokines and nitric oxide, and consequentially causes sympathetic dis regulation, alterations of endothelium and leukocyte organ infiltration. All these events were proposed to contribute to the induction of acute kidney injury AKI [2,4-6].

The endotoxin-induced inflammatory syndrome and related organ dysfunction are triggered by a variety of molecules. Mostly using animal models, a crucial role of CD44 has been demonstrated in mediating the progression of a variety of inflammatory diseases [7-11], including endotoxemia [12-15]. Conversely, some studies highlight an anti-inflammatory role of CD44 since it can contribute to the termination of inflammation by clearing apoptotic neutrophils and hyaluronan (HA) fragments from the site of injury [16,17] and by promoting the expression of negative regulators of TLR4 signaling [18-20].

CD44 is expressed on multiple cell types, including haematopoietic, mesenchymal, epithelial, and endothelial cells, and has several ligands, which differ per isoform [11,21]. CD44 transmembrane glycoproteins are encoded by a single gene of 20 exons, of which 11 are alternatively spliced "variant exons" (exons 6-15 and 19-20). The standard isoform, CD44s, is encoded by solely constant exons (exons 1-5 and 16-20), and is the shortest and most widely expressed isoform. Inclusion of the variant exons lengthens the extracellular domain (exons 1-17), creating larger isoforms and exposing binding sites for additional posttranslational modifications and ligand-binding sites [22]. CD44 glycoproteins mediate cell-responses to the cellular microenvironment, interacting with growth factors, chemokines, matrix metalloproteases and components of the extracellular matrix [21,23,24]. During inflammatory kidney diseases, renal CD44 expression, which is generally absent, is markedly enhanced, particularly in injured proximal tubular epithelial cells (TEC) [25-32]. In ischemia-reperfusion-mediated AKI, we previously found that lack of CD44 diminishes renal dysfunction, tubular damage and macrophage and granulocyte influx [28]. Until now, it remains unclear what are the implications of tubular CD44 expression during renal injury and moreover what are the differential functional properties of specific CD44 isoforms such as the shortest CD44s and the long-variant CD44-variant-3 (CD44v3, CD44v3-v10). The latter contains variant exons 3 to 10 and is a heparan sulfate (HS)-binding isoform of CD44 [21]. Both isoforms are induced in injured TEC and, having distinct extracellular domains, can exert diverse functions. Indeed, in a previous study, we showed that these two isoforms have opposite effect in fibrotic settings [29].

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CD44s is essential for some of the transforming growth factor (TGF)-β1-mediated actions [29-34]. Being a heparan sulfate proteoglycan, CD44v3 can interact with multiple molecules, such as hepatocyte growth factor (HGF) and vascular endothelial GF (VEGF) [21], and it might bind pro-inflammatory cytokines [11,35].

It is evident that the function of CD44 in inflammation is complex, involving multiple cell types, ligands, and intracellular pathways. It is therefore important to clarify the contribution of CD44 and of each isoform in inflammatory processes.

The present study aimed to assess the effects of renal tubular expression of CD44s and CD44v3 in SIRS-associated renal injury using wild-type (WT) and transgenic mice that express either CD44s or CD44v3 specifically on proximal TEC.

Methods

In vivo experimental design

Transgenic mice overexpressing either CD44s or CD44v3 specifically in proximal tubules are on a C57BL/6 background and were previously described [29]. In order to target proximal tubular cells, transgene expression was set under the control of the 5' regulatory promoter region of the γ-glutamyl transpeptidase type 1 gene [36].

Eight to 12 weeks old pathogen-free male C57BL/6 wild-type (WT) mice and CD44s/v3-transgenic mice (n=7-8 per group) were subjected to intraperitoneal injection of 10 μg/g body weight LPS (*Escherichia coli* O111:B4, Sigma-Aldrich). Sham mice received saline solution. Mice were sacrificed by cardiac exsanguination 4 and 24 hours after LPS injection and 4 hours after saline solution injection. Blood was drawn in heparinized tubes and half kidneys were snap-frozen in liquid nitrogen or fixed in 10% formalin. Mice body weight was measured at the time of injection and at the time of sacrifice. The Institutional Animal Care and Use Committee of the University of Amsterdam approved all animal experiments.

Immunohistochemistry, histological scoring and renal function

Renal tissues were fixed in 10% formalin for 24 hours and subsequently embedded in paraffin in a routine fashion. Immunohistochemistry stainings (immunostaining) were performed on 4 μm renal sections using anti-CD44 (IM7.8.1, BD Pharmingen), anti-active-caspase-3 (Cell Signaling), anti-CD3 (BD Pharmingen). Hyaluronan was detected by biotinylated HA-binding protein (Calbiochem). Slides were developed using HRP-labeled secondary antibody (DAKO) and DAB (Sigma-Aldrich). As previously described, quantification of immunohistochemistry stainings was assessed in the cortex and cortico-medullary area by counting positive cells per high power field (HPF, x40 magnification) or by measuring the positive areas in 10-15 HPF (x20) pictures per slide using ImageJ software (National Institute of Health, US) [29]. Results are shown as positive area in percentage of the total area analyzed. For assessing renal function, plasma urea concentration was measured by standard diagnostic procedure suitable for detection of samples of murine origin.

ELISA

Heparinized blood was centrifuged at 10000 rpm for 10 minutes and plasma was harvested. Frozen kidneys were homogenized in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl_2$ pH 7.4, 1 mM $CaCl_2$, 1% Triton) with addition of 1% protease inhibitor cocktail (P8340, Sigma). Specific ELISAs (R&D Systems) were utilized to measure MCP-1, TNF-α, IL-1β, IL-10, HGF, and TGF-β1 in plasma or kidney homogenates, according to the manufacturer instructions.

Quantitative Real-time PCR

Total RNA was extracted from 10 frozen renal sections (30 μm thick) with Trizol reagent (Invitrogen). RNA was converted to cDNA using oligo-dT primers. Quantitative real-time PCR (Q–PCR) was performed on a LightCycler® 480 System (Roche) using SYBR Green-SensiMix (Bioline). SYBR green dye intensity was analyzed with linear regression analysis. Transcript expression was normalized towards the housekeeping gene TATA-box binding protein (TBP). Amplified genes and primers sequences are as follows: TBP forward (F) 5'-caggagccaagagtgaagaac reverse (R) 5'-ggaaataattctggctcatagctact, CD44-pan F 5'-tccgaattagctggacactc R 5'-ccacaccttctcctactattgac, CD44s F 5'-ttctggaatctgaggtctcc R 5'-caccttggccaccagagatcg, CD44v3 F 5'-catcatcaatgcctgatcca R 5'-agtcaaataccaacccaacag, TLR4 F 5'-ggactctgatcatggcactg R 5'-ctgatccatgcattggtaggt, kidney injury molecule-1 (KIM-1) F 5'-tggttgccttccgtgtctct R 5'-tcagctcgggaatgcacaa, neutrophil gelatinase-associated lipocalin (NGAL) F 5'-gcctcaaggacgacaacatc R 5'-ctgaaccattgggtctctgc, A20 F 5'-gggactccagaaaacaaggg R 5'-taccttcaaacatggtgctt, interleukin-1 receptor-associated kinase-3 (IRAK-3) F 5'-catctgtggtacatgccagaa R 5'-acctccatgatcacgattcc, suppressor of cytokine signaling-1 (SOCS-1) F 5'-gacactcacttccgcacctt R 5'-aagaagcagttccgttggc. All primers were manufactured by Biolegio.

Data analysis

Kruskal-Wallis analysis and Dunn's multiple comparisons test were applied to evaluate statistically significant differences among the three mice strains. Mann-Whitney U test was used to compare LPS-injected group to its respective control sham group. Data are shown as mean and standard error of the mean (SEM); $p<0.05$ was considered to be significant.

Results

Systemic effects and renal CD44 expression

SIRS was equally induced in all mice upton LPS administration. All mice displayed a significant body weight loss after 24 hours (Supplementary Figure 1A), and plasma levels of MCP-1 and TNF-α were found upregulated 4 hours after injection in comparison to sham animals (Supplementary Figure 1B and C). Surprisingly, TNF-α plasma levels were higher at 4 hours in CD44v3-mice in respect to WT (Supplementary Figure 1C). Since in others renal pathological conditions, CD44 expression is induced in the kidney parenchyma [27-29,31,32,37], we assessed renal expression of CD44 by Q-PCR and immunostaining. Q-PCR analysis showed that CD44 transcripts were upregulated at 4 and 24 hours in all mice groups in respect to sham (Figure 1A). Isoform-specific Q-PCR (Figure 1B and C) and immunostaining for CD44 (Figure 1D) revealed that solely CD44s or CD44v3 were overexpressed in sham kidneys of transgenic mice and their expression was restricted to the basolateral membrane of proximal TEC [29]. After LPS administration, CD44-positive cells were localized in the interstitium and glomeruli in WT kidneys and also at the basolateral membrane of TEC in kidneys of transgenic mice (Figure 1D).

A strong expression of CD44 on renal tubules, glomeruli and interstitial cells was found in a kidney biopsy derived from a septic patient who developed acute renal insufficiency (Supplementary Figure

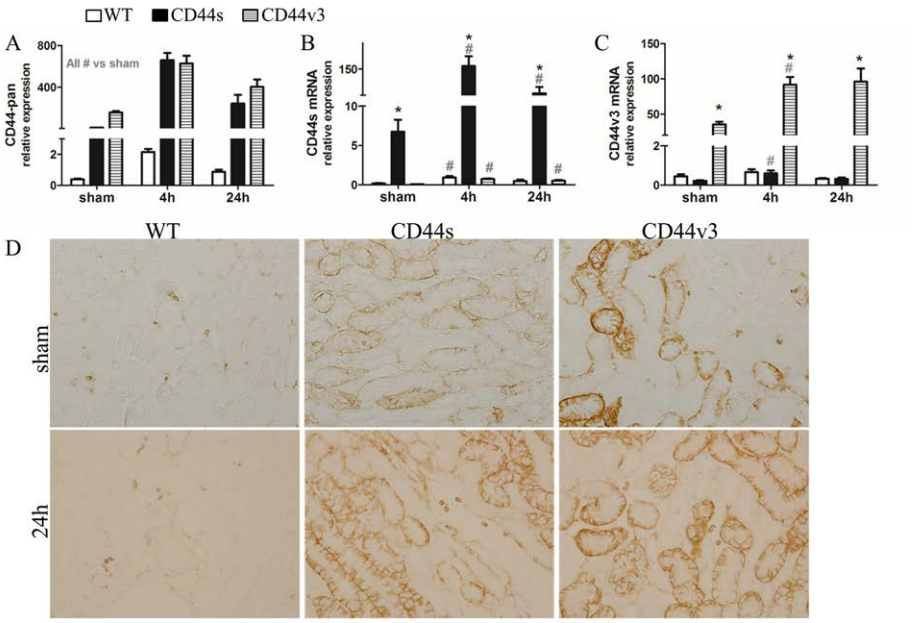

Figure 1: (A) Q-PCR analysis for CD44-pan, (B) CD44s, and (C) CD44v3 expression in kidneys of WT (white bars), CD44s- (black bars) and CD44v3-transgenic mice (stacked bars). Data normalized for TBP expression levels. (D) Representative micrographs (x20) of CD44 immunostaining in renal paraffin sections of WT and CD44s- and CD44v3-transgenic mice in sham condition and 24h after LPS-injection. Mean + SEM, n=7-8, *=p<0.05, #=p<0.05 vs sham.

Renal function and tubular damage

It has been previously reported that LPS causes mild morphological changes in renal tissues, including tubular cell sloughing and loss of brush border [2,6]. These minimal histological changes were visible 24 hours after LPS administration and similar in all groups (Figure 2A). Renal function was assessed by measuring plasma urea levels (Figure 2B). Blood urea levels were significantly increased at 4 hours and picked at 24 hours in all mice strains as compared to sham. At 4 hours, CD44v3-mice showed a small but significant reduction in plasma urea levels as compared to WT mice.

Next, we evaluated the mRNA expression of the early markers of tubular injury KIM-1 and NGAL [38], which gradually increased in time after LPS administration in all strains (Figure 2C and D). The overexpression of both CD44s and CD44v3 resulted in a higher expression of KIM-1, which is a marker specifically induced in proximal tubules after damage [39]. No significant differences among the strains were found in the levels of NGAL, which is expressed in the distal nephron segments [40].

Finally, we assessed the apoptosis rate of tubular cells after LPS challenge. The number of apoptotic TEC increased in time during endotoxin shock, but was however minimal and similar among the groups (Figure 2E).

Renal inflammatory cell influx and cytokine release.

During SIRS, the initial cytokine storm leads to upregulation of endothelial adhesion molecules and, hence, leukocyte extravasation and amplification of imflammation in multiple organs [3]. Immunohistochemistry staining for CD3 revealed an equal influx of lymphocytes into kidneys of all 3 mice strains (Figure 3A).

Next, we measured the levels of pro-inflammatory (MCP-1, TNF-α, IL-1β) and anti-inflammatory (IL-10, HGF, TGF-β1) cytokines produced in the kidneys upon LPS administration (Figure 3B-G). As expected, all cytokines were upregulated in kidneys of LPS-treated mice, although some differences were found in the levels of TNF-α, IL-1β at 4 hours with less expression in the CD44s- and CD44v3-kidneys as compared to WT kidneys. Interestingly, at 24 hours IL-10 production remained high in the CD44v3-kidneys, whereas it decreased to basal levels in WT and CD44s-kidneys.

Successively, the induction of the endogenous negative regulators of the TLR4/NF-κB pathway [41] was evaluated by Q-PCR analysis (Figure 3H-J). Gene expression of A20, IRAK-3 and SOCS-1 was greatly increased at 4 hours in all groups in a similar manner. Strikingly, at 24 hours the presence of CD44v3 on TEC resulted in significantly higher levels of the three regulators in respect to WT and CD44s-kidney levels, in accordance to the IL-10 expression pattern. It is to be noted that the expression of TLR4 mRNAs in the kidneys was similar among the strains before and after LPS treatment (Figure 3K).

Discussion

Sepsis is a major public health problem being the leading cause of death in non-coronary intensive care units [42]. Development of AKI during sepsis is associated with prolonged hospitalization and a higher mortality rate (70% versus 45% rate of acute renal failure alone) [2,43]. It remains therefore imperative to better understand the pathophysiology of sepsis and SIRS-associated AKI and to identify new targets in order to improve septic shock treatment.

Here, we studied the potential role of CD44s and CD44v3 expression by TEC in the progression of SIRS-induced AKI. Although, the presence of CD44 isoforms did not substantially alter the onset of

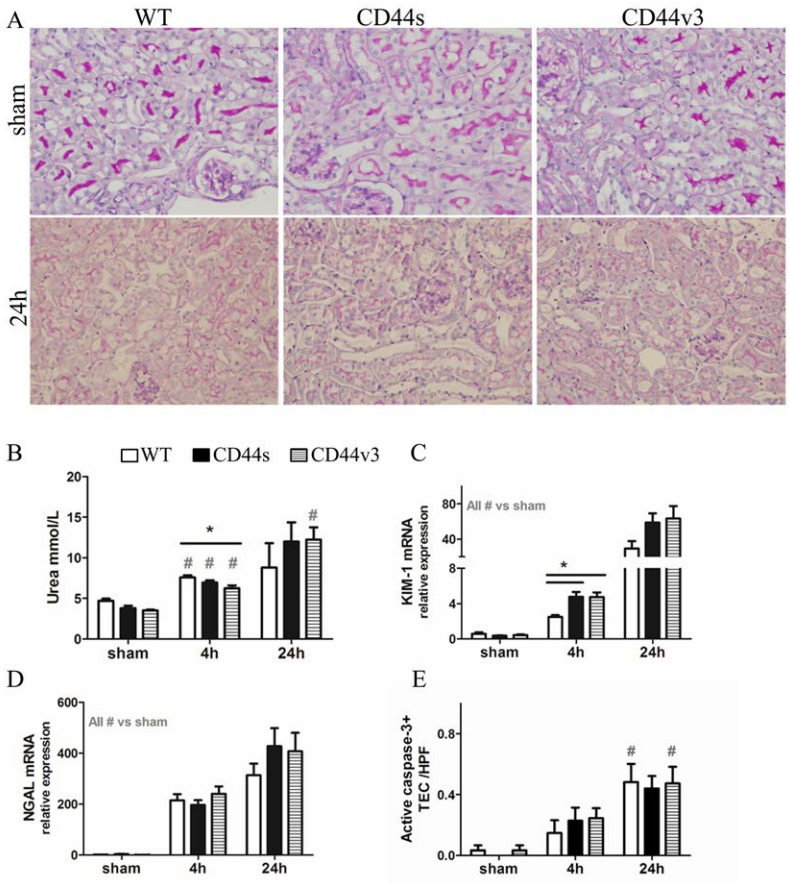

Figure 2: (A) Representative micrographs (x20) of PAS-D stained renal sections of sham and 24h-LPS treated animals. (B) Renal function assessed by measurement of urea levels (mmol/L) in plasma of WT (white bars), CD44s- (black bars) and CD44v3-transgenic mice (stacked bars). (C) Q-PCR analysis for KIM-1 and (D) NGAL gene expression. Values corrected for TBP transcript levels. (E) Quantification of tubular cell apoptosis rate by counting active caspase-3+ TEC per HPF (x40). Mean + SEM, n=7-8, *=p<0.05, #=p<0.05 vs sham

kidney dysfunction upon LPS injection, their expression influenced the induction of KIM-1 and the levels of pro- and anti-inflammatory cytokines in kidneys after LPS treatment.

SIRS was equally triggered by intraperitoneal injection of LPS in all mice strains; consequently, similar numbers of lymphocytes were found in the kidneys of LPS-treated mice. The difference in blood levels of TNFα between CD44v3- and WT-mice at 4 hours appears not to be particularly relevant for the renal outcome as it does not associate with the rate of renal infiltrates, cytokines, or renal dysfunction.

The changes induced by LPS injection in the kidneys are mainly vascular, whereas tubules undergo slight vacuolization and loss of brush border and few TEC undergo apoptosis [2,6]. Presumably due to these minimal tubular changes, CD44 expression was not induced in tubules of murine WT kidneys upon LPS injection. This contrasts what is reported for other renal inflammatory disorders, such as human IgA nephropathy [27], and renal transplants [31,32], murine renal ischemia-reperfusion injury [28], unilateral ureteric obstruction injury [29,44], interstitial nephritis [30], and lupus nephritis [26]. We cannot anyhow exclude an induction of renal tubular CD44 expression in human cases of severe sepsis with serious renal complications. In a kidney biopsy from a patient with tubulonecrosis and AKI subsequent to streptococcal septicemia, CD44 was found expressed in renal tubules, glomeruli and interstitial cells; unfortunately, this was the only renal biopsy of SIRS patients present in our tissue bank.

In the early acute phase of SIRS, WT tubular cells, which lack CD44, displayed lower levels of KIM-1 in comparison to TEC overexpressing CD44s/v3. KIM-1 expression levels did not correlate with kidney dysfunction, which was similar among the different groups. KIM-1 and NGAL are sensitive biomarkers of renal injury and their expression precedes kidney dysfunction [38]. The fact that only KIM-1 and not NGAL shows different expression levels in the transgenic mice as compared to WT animals can be attributed to its expression in the proximal tubules [39], whereas NGAL is mainly expressed in the distal part of tubules [40]. Given the similarity in blood urea levels among the strains, it remains difficult to explain the higher expression of KIM-1 in the transgenic mice at 4 hours. As transgenes are expressed solely in

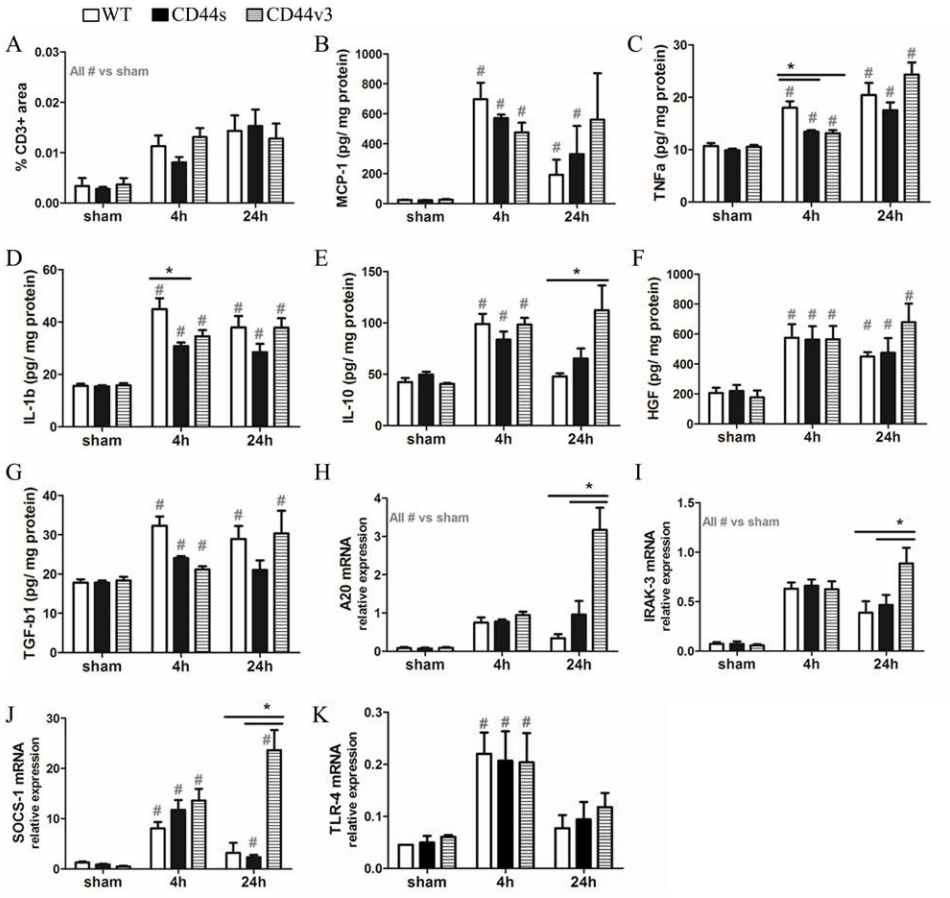

Figure 3: Renal inflammation. (A) Evaluation of lymphocyte influx by digital image analysis of CD3-stained paraffin renal sections from WT (white bars), CD44s- (black bars) and CD44v3-transgenic mice (stacked bars). Data expressed as percent positive area of the total areal analyzed. (B) Renal expression (pg/mg proteins) of MCP-1, (C) TNF-α, (D) IL-1β, (E) IL-10, (F) HGF and (G) TGF-β1. (H) Gene expression of A20, (I) IRAK-3, (J) SOCS-1 and (K) TLR4 in kidneys, assessed by Q-PCR assays. Values corrected for number of TBP transcripts. Data expressed as mean + SEM, n=7-8, *=p<0.05, #=p<0.05 vs sham.

proximal tubules, it is reasonable to conclude that the dissimilarities in renal cytokine levels are mediated by diverse behavior of the proximal TEC and not by inflammatory cells, which are identical in WT and transgenic mice. This implicates an active action of proximal TEC during SIRS-induced AKI. In culture, primary tubular cells have been shown to respond to LPS by inducing cytokine expression [45,46].

CD44v3 facilitates HGF-receptor binding and signaling [21,47]; we and others showed a link between CD44s expression and higher TGF-β1/Smad-signaling [29,33,34]. In this model, both HGF and TGF-β1 are beneficial as both molecules can exert anti-inflammatory effects. Thus, the facilitation of HGF and TGF-β1 signaling pathways by CD44v3 and CD44s, respectively, explain, at least in part, the lessened production of pro-inflammatory cytokines in the kidneys of transgenic mice.

Surprisingly, 24 hours post-injection, CD44v3-kidneys displayed an elevated rate of the anti-inflammatory cytokines IL-10 and HGF, together with an increased gene expression of the negative regulators of the TLR4 signaling.

Previous studies described CD44 as a negative regulator of inflammation due to induction of TLR4 signaling inhibitory molecules [18-20]. We can speculate that this anti-inflammatory function is exerted at least by the CD44 variant-3 isoform, as the presence of CD44s did not result in higher levels of A20, IRAK-3 or SOCS-1. Muto et al. showed that hyaluronan (HA) can induce A20 expression via CD44 and TLR4 [20]. Although all CD44 isoforms have a HA-binding site, the binding affinity to HA differs per isoform and cell-type, and is regulated by posttranslational modification: e.g. addition of heparan sulfates decreases the CD44-mediated binding to hyaluronan [48]. Hence, HA-CD44 interaction is not likely to be the main mechanism of induction of TLR4 negative regulators, which are highly expressed only in CD44v3-kidneys and not in CD44s-kidneys, besides similar levels of renal interstitial hyaluronan between the two transgenic strains (data not shown).

Although induced by LPS, interleukin-10 provides protection

against LPS-induced septic shock [49]. IL-10 inhibits the rate of transcription of LPS-induced inflammatory genes and triggers the expression of NF-κB-inhibitory molecules [50], providing a plausible explanation for what occurs in the CD44v3-kidneys at 24 hours. These anti-inflammatory effects have been shown to be mediated in part by STAT-3 and heme oxygenase-1 (HO-1) [49,50], both of which in turn can mediate IL-10 upregulation [51,52]. HO-1 is induced by HGF and provides protection against endotoxemia [52,53]. We could hypothesize that CD44v3, via binding HGF and facilitating its signaling, could cause HO-1 upregulation, and hence production of IL-10, which in turn triggers, through STAT-3/HO-1, expression of TLR4/NF-κB pathway-inhibitory molecules. Interestingly, Lee et al. demonstrated that, once internalized and translocated into the nucleus, CD44 forms a complex with STAT3 and p300 acetyltransferase, eliciting STAT3 acetylation and its consequential activation [54].

Beyond sepsis/SIRS studies, many reports demonstrated a crucial role of CD44 in inflammation and the beneficial effects of anti-CD44 therapies in several inflammatory diseases, including renal ischemia-reperfusion injury [28], collagen- and proteoglycan-induced arthritis [7], cutaneous inflammation [8], experimental autoimmune encephalomyelitis [9], and IL-2-induced vascular leak syndrome [10]. The mentioned studies targeted mainly CD44 on leukocytes.

CD44 is expressed by a variety of cells, and its functions differ depending on the cell-type and/or ligand. Our results suggest that proximal tubular cells can directly influence the renal inflammatory milieu and propose a role of CD44v3 in limiting renal inflammation.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

E.R., J.C.L and S.F. conceived the study design and were involved in interpreting the data, writing and reviewing the manuscript. E.R., N.C. and G.J.D.T. carried out the experiments, analyzed, interpreted the data, and provided intellectual content to the manuscript. All authors gave input to and approved the final manuscript.

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