Toll-like receptor 4 signalling mediates inflammation in skeletal muscle of patients with chronic kidney disease

Daniela Verzola, Alice Bonanni, Antonella Sofia, Fabrizio Montecucco, Elena D'Amato, Valeria Cademartori, Emanuele Luigi Parodi, Francesca Viazzi, Chiara Venturelli, Giuliano Brunori & Giacomo Garibotto*

Department of Internal Medicine, Nephrology Division and First Clinic of Internal Medicine, University of Genova and IRCCS AOU San Martino-IST, and Ospedale Santa Chiara[1], Trento, Italy

Background   Inflammation in skeletal muscle is implicated in the pathogenesis of insulin resistance and cachexia but why uremia up-regulates pro-inflammatory cytokines is unknown. Toll-like receptors (TLRs) regulate locally the innate immune responses, but it is unknown whether in chronic kidney disease (CKD) TLR4 muscle signalling is altered. The aim of the study is to investigate whether in CKD muscle, TLRs had abnormal function and may be involved in transcription of pro-inflammatory cytokine.

Methods   TLR4, phospho-p65, phospho-ikBα, tumour necrosis factor (TNF)-α, phospho p38, Murf 1, and atrogin were studied in skeletal muscle from nondiabetic CKD stage 5 patients ($n = 29$) and controls ($n = 14$) by immunohistochemistry, western blot, and RT–PCR. Muscle cell cultures (C2C12) exposed to uremic serum were employed to study TLR4 expression (western blot and RT–PCR) and TLR-driven signalling. TLR4 signalling was abrogated by a small molecule chemical inhibitor or TLR4 siRNA. Phospho AKT and phospho p38 were evaluated by western blot.

Results   CKD subjects had elevated TLR4 gene and protein expression. Also expression of NFkB, p38 MAPK and the NFkB-regulated gene TNF-α was increased. At multivariate analysis, TLR4 protein content was predicted by eGFR and Subjective Global Assessment, suggesting that the progressive decline in renal function and wasting mediate TLR4 activation. In C2C12, uremic serum increased TLR4 as well as TNF-α and down-regulated pAkt. These effects were prevented by blockade of TLR4.

Conclusions   CKD promotes muscle inflammation through an up-regulation of TLR4, which may activate downward inflammatory signals such as TNF-α and NFkB-regulated genes.

Keywords   Chronic kidney disease; Toll-like receptors; Muscle wasting; Inflammation; Tumour necrosis factor-α

Introduction

Although uremic cachexia has for a long time been regarded as a non-immune disease [see Ref. 1 for Review], an emerging hypothesis is that innate immunity plays a role in its development and progression,[1–3] in analogy with cancer[4] and cardiac[5] cachexia. Initial observations have shown that circulating levels of C-reactive protein (CRP) and proinflammatory cytokines, including interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), are increased in blood of patients with CKD and are strongly associated with wasting.[6,7] More recently, an up-regulation of several genes associated with inflammation in muscle has been demonstrated to occur both in rodents models[8,9] and humans with CKD.[10–12] Pro-inflammatory cytokines, mainly TNF-α and IL-6, have *per se* catabolic effects on skeletal muscle by accelerating protein degradation or reducing protein synthesis.[13–15] In addition, in CKD proinflammatory cytokines cause a resistance to the action of insulin[16] and GH/IGF-1.[17,18] Bailey *et al.* recently identified a series of abnormal postreceptor signalling changes in the insulin/IGF-1 pathway leading to atrophy in muscle of rats with CKD.[19] These include the occurrence of functional abnormalities in the IRS/PI3-K cascade that decrease the phosphorylation of the downstream effector Akt and the stimulation of the expression of specific E3 ubiquitin conjugating enzymes, atrogin-1/MAFbx and MuRF1

with acceleration of protein degradation.[19,20] Of note, we could observe that the pAkt signal is markedly down-regulated in muscle of patients with advanced CKD and is associated with apoptotic cell loss, suggesting that insulin resistance is a major player in the regulation of muscle cell survival and catabolism in renal patients.[12] In addition, Zhang *et al.* could recently demonstrate that high IL-6, by the activation of the JAK-Stat3 pathway, up-regulates the gene expression of myostatin, a major negative regulator of muscle protein content and regeneration. Such a mechanism has been observed both in the rodent model and in muscle of CKD patients.[9] Interestingly, Myiamoto *et al.*[21] observed that increased circulating follistatin, which neutralizes the biologic activities of myostatin, occurred together with inflammation and reduced muscle strength in a large cohort of stage V CKD patients, suggesting an involvement of the myostatin regulation system in the uremic wasting process.

Despite our increased understanding of mechanisms by which systemic inflammation regulates the pathways leading to muscle catabolism in uremia, the reason(s) why uremia up-regulates pro-inflammatory cytokines in skeletal muscle is unknown. Skeletal muscle possesses both the afferent and efferent limbs of the innate immune system, including Toll-like receptors (TLRs) and both early- and late-phase cytokines.[22–24] TLRs are a family of receptors in the innate immune system which mediate signal transduction pathways through the activation of transcription factors that regulate the expression of proinflammatory cytokines in several cell types and tissues.[22] In skeletal muscle, TLRs act as a sentinel to monitor for the presence of pathogens[23] and, upon activation, induce a local inflammatory response[24,25] culminating in the translocation of NFkB to the nucleus and activation of inflammatory genes, including TNF-α, IL-1ß, and IL-6. In addition to microbial products, TLRs can also be activated by endogenous signals of tissue injury, including debris from apoptotic and necrotic cells, oligosaccharides, heat shock proteins, and nucleic acid fragments.[26,27]

In this study we hypothesize that CKD patients have abnormal function of TLRs in muscle and that TLRs may be involved in initiating events associated with the stimulation of muscle pro-inflammatory cytokine transcription. We tested this postulate by different measures. First we studied TLR gene and protein expression in muscle biopsies of patients with CKD and compared the results to those obtained in subjects with normal renal function. As a second step, to identify specific TLR-linked transcriptional pathways we studied the expression profiles of selected TLR4 downward genes and molecules in uremic muscle. As a third step, we studied the clinical correlations associated with muscle TLR4 expression and we observed that TLR4 protein expression is inversely related to residual renal function, suggesting that more advanced uremic state activates muscle TLR4. Additionally, we studied the response to uremic serum of C2C12 myotubes and we observed that uremic serum up-regulates

TLR4 and TNF-α expression and down-regulates pAkt. Such effects are prevented by TLR4 inhibitors or TLR4 knockdown. Overall, our data demonstrate the activation of TLR4 and its downward inflammatory cascade in muscle of subjects with CKD and suggest that enhanced TLR4 signalling contributes to the up-regulation of native immunity in skeletal muscle in uremia.

Methods

Study participants

Starting from 5 September 2005, 29 nondiabetic CKD patients (18 M/11 F) scheduled for peritoneal dialysis catheter insertion were eligible for enrolment in this protocol at the Nephrology Division, Department of Internal Medicine, University of Genoa (Table 1). The study was part of a protocol on the effects of peritoneal dialysis on protein turnover approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa. All subjects were informed about the nature, purposes, procedures, and possible risks of the study, before their informed consents were obtained. The procedures were in accordance with the Helsinki declaration regarding ethics of human research. Patients younger than 18 and older than 85 years were excluded from the study. The patients were enrolled in the study on a consecutive basis if they did not meet the following exclusion criteria: New York Heart Association

Table 1 Clinical characteristics of controls and chronic kidney disease (CKD) subjects

	Controls	CKD subjects
Number of subjects	14	29
Age (years)	64 ± 5	67 ± 2
Gender (M/F)	9/5	18/11
BMI (kg/m^2)	25 ± 1	25 ± 1
FFM (kg)	46 ± 2	43 ± 3
Fat mass (kg)	25 ± 1	26 ± 2
nPNA (g/kg)	1.0 ± 0.1	0.93 ± 0.1
SGA score	7 (6–7)	5 (2–7)
CRP (mg/L)	3 (2–4)	8 (2–27)[a]
Estimated GFR (mL/min.1.73 m^2)	99 ± 6	8 ± 0.7[b]
[HCO$_3$] (mmol/L)	24 ± 1	23 ± 1
Albumin (g/dL)	4.2 ± 0.1	4.0 ± 0.2
Haemoglobin (g/dL)	13 ± 1	11.3 ± 0.2[a]
BUN (mg/dL)	17 ± 2	90 ± 4[b]
Calcium (mg/dL)	9.5 ± 0.3	8.5 ± 1
Phosphate (mg/dL)	4.1 ± 0.1	5.9 ± 0.4[b]
iPTH (pg/mL)	na	408 ± 22[b]

Data are mean ± SEM or median (range). Abbreviations: BMI, body mass index; CRP, C-reactive protein; FFM, fat-free mass; GFR, glomerular filtration rate; na, not available; nPNA, normalized protein nitrogen appearance; SGA, subjective global assessment. Significance of difference vs. control subjects: a = $P < 0.05$; b = $P < 0.01$.

Class III–IV congestive heart failure, a recent (<12 months) myocardial infarction, liver cirrhosis, infection, or diabetic nephropathy. Clinical and biochemical characteristics of the subjects are shown in Table 1. All groups were closely matched for age and gender. All subjects had a sedentary life style. Their mean age was 67 years (range 43–82 years). Their estimated[28] GFR was 8 ± 1 mL/min 1.73 m^2 (range 4–14 mL/min).

Causes of renal diseases were hypertensive nephrosclerosis (11 patients), chronic glomerulonephritis (8 patients), tubulointerstitial nephritis (3 patients), polycystic kidney disease (6 patients), and obstructive uropathy (1 patient). Their mean estimated protein and calorie intake were 0.9 g/kg and 27 kcal/kg, respectively. Their nutritional status was measured with the 7-point Subjective Global Assessment (SGA) index.[29,30] By this approach, an overall SGA classification of 1–7 is assigned; a score of 7 indicates a normal nutritional status and a score of 1 indicates severe protein-energy wasting. Albumin levels were low (<3.8 g/100 mL) in 14 subjects, while BMI was low (<23 kg/m^2) in six subjects. The evidence of an inflammatory response (CRP >5 mg/L) was shown in 11 subjects.

Mean muscle fibre cross-sectional area (CSA) was lower in patients with CKD (median = 976 μm^2, range 745–1615; controls median = 1422 μm^2, range 1100–1974) ($P < 0.05$), suggesting muscle atrophy. CKD subjects were more insulin resistant than control subjects, based on a higher HOMA index and plasma insulin concentration ($P < 0.05$).

Control biopsies were taken in 14 otherwise healthy subjects, during elective surgery for abdominal wall hernias. Controls were selected on the basis of having no chronic illnesses or acute inflammatory processes. In these subjects baseline physical examination and eGFR (99 ± 6, range 78–120 mL/min) as well as screening biochemical tests of renal, hepatic, haematological, and metabolic function (thyroid function and fasting plasma glucose) were unremarkable.

Muscle biopsies

Muscle biopsies were obtained from *rectus abdominis* muscle, at the beginning of surgery. Tissue (90 mg) RNA was isolated using the Qiazol Lysis reagent (Qiagen Sciences, Maryland, USA). Isolated RNA was stored at −80 °C until use. Other sample aliquots were used for immunohistochemical staining, and for protein (western blot) analysis. For the study of fat infiltration, tissue samples were stained with Oil-red-O, which detect lipids, mainly neutral fats and cholesteryl esters (Sigma-Aldrich, Milan, Italy) (31). Digital images of immunohistochemical and Oil-red-O stained sections were obtained using a Leica microscope (Leica Microsystems GmbH Wetzlar, Germany) equipped with a digital camera controlled by Q500MC Software-Qwin (Leica).[12]

Tissue analyses

Histological preparation, immunohistochemical staining, and Western blot were performed as previously described.[12] mRNAs were analysed by RT–PCR as described [53]. Primers are listed in Table 2. Relative mRNA levels were calculated from cycle threshold (Ct) values using β-actin as the internal control.

Cell culture and treatments

Mouse skeletal muscle cell line C2C12 was propagated as myoblasts in DMEM (Euroclone, Milan, Italy) containing 2 mmol L-glutamine and 100 U/mL penicillin-streptomycin (Euroclone), with 5% FBS and incubated at 37 °C. For differentiation into myotubes, the myoblasts at 90% confluence were incubated with DMEM plus 2% horse serum (Sigma Chemical Co, St. Louis, MO, U.S.A.). The myotubes began to form in 2–4 days, and multinucleated muscle fibre cultures were used at 7 days. For evaluating TLR 4 induction, myotubes were incubated with 10% pooled human normal serum or pooled human uremic serum.

Experimental conditions

C2C12 mouse myotubes were incubated in the presence of uremic serum or normal serum for 2, 4, and 6 h to assess TLR4 and PKC expression. TLRs expression was studied by

Table 2 Primer sequences for RT–PCR analyses

Primers	Forward	Reverse
TLR4 (human)	AGCCACGCATTCACAGGG	CATGGCTGGGATCAGAGT CC
TNF-α (Human)	AGGTTCTCTTCCTCTCACAT	ATCATGTTTCAGTGCTCATG
β-Actin (Human)	CATCCCCCAAAGTTCACAAT	AGTGGGGTGGCTTTTAGGAT
Atrogin1/MAFbx (human)	TTTCCTGGAAGGGCACTGAC	ACGACTGACCTCTCGACCCTTAT
MURF1 (human)	GCCACCTTCCTCTTGACTG	ATTCTTCCTCTTCATCTGTC
TLR4 (mouse)	AGCTTCTCCAATTTTTCAGAACTTC	TGAGAGGTGGTGTAAGCCATGC
TNF-α (mouse)	CACGCTCTTCTGTCT ACTGA	GGACTCCGTGATGTCTAAGT
GAPDH (mouse)	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGAT CA

western blot. In selected experiments, TLR4 and PKC receptor antagonists were added to the cells 1 h before uremic serum stimulation. C2C12 were blocked for 2 h before uremic serum treatment with 30 μM VIPER (viral inhibitory peptide of TLR4) or CP7 (inert control peptide, designed as negative control for inhibitory assay). Cells were exposed to uremic serum for 5 h and then total RNA was extracted using TRIzol, reverse transcribed, and TNF-α mRNA expression was analysed by real time PCR, as above described.

Uremic serum

Uremic serum was collected from patients with ESRD on hemodialysis recruited randomly from a pool of 150 patients at the Nephrology Division at the Genoa University. Patients were recruited over a 2 month period. Informed consent was obtained, and 3 mL of blood was collected before the next subsequent hemodialysis. Healthy age-matched donors were used as controls. Blood urea nitrogen, creatinine, and glucose were assayed in all patients, and control sera were excluded if creatinine was >1.0 mg/dL. Exclusion criteria were presence of inflammatory disease, acute or chronic infection, autoimmune or liver diseases, diabetes, and malignancy. None of the patients or controls smoked. Blood was collected in Vacutainer tubes, and serum was separated by centrifuging clotted blood at 1100 g for 10 min at room temperature to obtain serum. In order to minimize minor differences between patients, all serum samples were pooled for the experiments. Serum samples were frozen at −20 °C until analysis. In preliminary experiments, 10% was found to be the highest nontoxic concentration of uremic serum.

Systemic inflammatory marker detection in uremic serum

Cytokine levels in uremic serum are shown in Table 3. Serum CRP, osteoprotegerin (OPG), TNF-a, CCL2, adiponectin, leptin,

Table 3 Cytokine levels in normal and uremic serum

	Control serum	Uremic serum
TNF-α (pg/mL)	9.7	15.6
CRP (mg/L)	0.73	11.9
s-IL6 receptor (ng/mL)	32.1	56.2
IL-6 (pg/mL)	9.0	52.0
Endotoxin EU/mL	0.6	1.0
MCP1 (pg/mL)	52.2	67.3
Adiponectin (microg/mL)	3.1	9.1
Leptin (ng/mL)	10.7	43.1
OPN (ng/mL)	46.2	126.5
Resistin (ng/mL)	15.9	36.3
OPG (pg/mL)	920	5443

Abbreviations: CRP, C-reactive protein; IL6, interleukin-6; MCP1, monocyte chemotactic protein 1; OPG, osteoprotegerin; OPN, osteopontin; TNF-α, tumour necrosis factor α.

resistin, IL-6, soluble IL-6 receptor (s-IL-6R), and osteopontin (OPN) complex levels were measured by colorimetric enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Minneapolis, Minnesota, USA), following manufacturer's instructions. Mean intra- and inter-assay coefficients of variation (CV) were below 8% for all markers. Serum lipopolysaccharide quantification was performed using a Limulus Amebocyte assay (Cambrex, Verviers, Belgium).

Block of Toll-like receptor 4 by viral inhibitory peptide of Toll-like receptor 4

C2C12 were blocked, for 2 h before uremic serum treatment, with 30 μM VIPER (viral inhibitory peptide of TLR4) or CP7 (inert control peptide, designed as negative control for inhibitory assay) (Imgenex, San Diego, CA). Cells were exposed to uremic serum for 5 h and then total RNA was extracted using TRIzol, reverse transcribed, and TNF-α mRNA expression was analysed by real time PCR, as above described.

RNA interference

C2C12 were transfected with 60 nM TLR4 specific siRNA or negative control siRNA (Ambion, Carlsbad, CA) using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and then incubated at 37 °C in a CO_2 incubator for 24 h until the cells were ready for assay. The efficacy of knockdown was determined by real-time PCR.

Statistical analysis

All data are presented as the mean ± standard error of the mean or median (range). Specific mRNAs were normalized for the internal control gene (β-actin) and are expressed as transcript/housekeeping gene ratios. The control treated group mean was given a value of 100, and individual values are expressed relative to this value. Statistical analysis was performed by using the SPSS statistical package (version 16; SPSS, Chicago, IL). For statistical analysis of expression variables that did not have a Gaussian distribution, values were logarithmically transformed or analysed by nonparametrical tests. Comparisons between groups were performed by one-way ANOVA with a *post hoc* Bonferroni correction or by a Kruskal–Wallis nonparametric test when appropriate. A multivariate regression model was created to study the predictors of TLR4 content in muscle. Statistical significance occurred if a computed two-tailed $P < 0.05$.

Results

Elevated Toll-like receptor 4 gene expression and protein content in muscle of patients with chronic kidney disease

Figures 1 and 2 show TLR2, TLR3, and TLR4 mRNA and protein levels in muscle of controls and CKD patients. While TLR2 and TLR3 were normally regulated (Figure 1), TLR4 mRNA was significantly overexpressed (by approximately two-fold; $P < 0.05$) in muscle of CKD subjects compared with controls (Figure 2A). Consistent with the increases in TLR4 gene expression, CKD subjects had higher TLR4 muscle protein content than control subjects ($P < 0.05$–0.001) as shown by immunohistochemistry and western blot (Figure 2B–D).

Toll-like receptor 4-driven signalling in skeletal muscle of patients with chronic kidney disease

As a next step, we examined whether increased TLR4 expression in CKD subjects was associated with abnormal TLR4 signalling. As

Figure 1 Expression of TLR2 (a) and TLR3 (b) mRNAs and proteins in skeletal muscle of controls ($n = 14$) and patients with chronic kidney disease (CKD) ($n = 29$). TLR2 and TLR3 mRNA expression was determined by real-time PCR and their protein expression by immunohistochemistry and image analysis. Values are expressed as fold increase ± SEM to the control muscle. TLR2 and TLR3 mRNAs and proteins were unchanged with respect to control subjects ($P = $ NS). CKD = chronic kidney disease. (Magnification: ×400–1000).
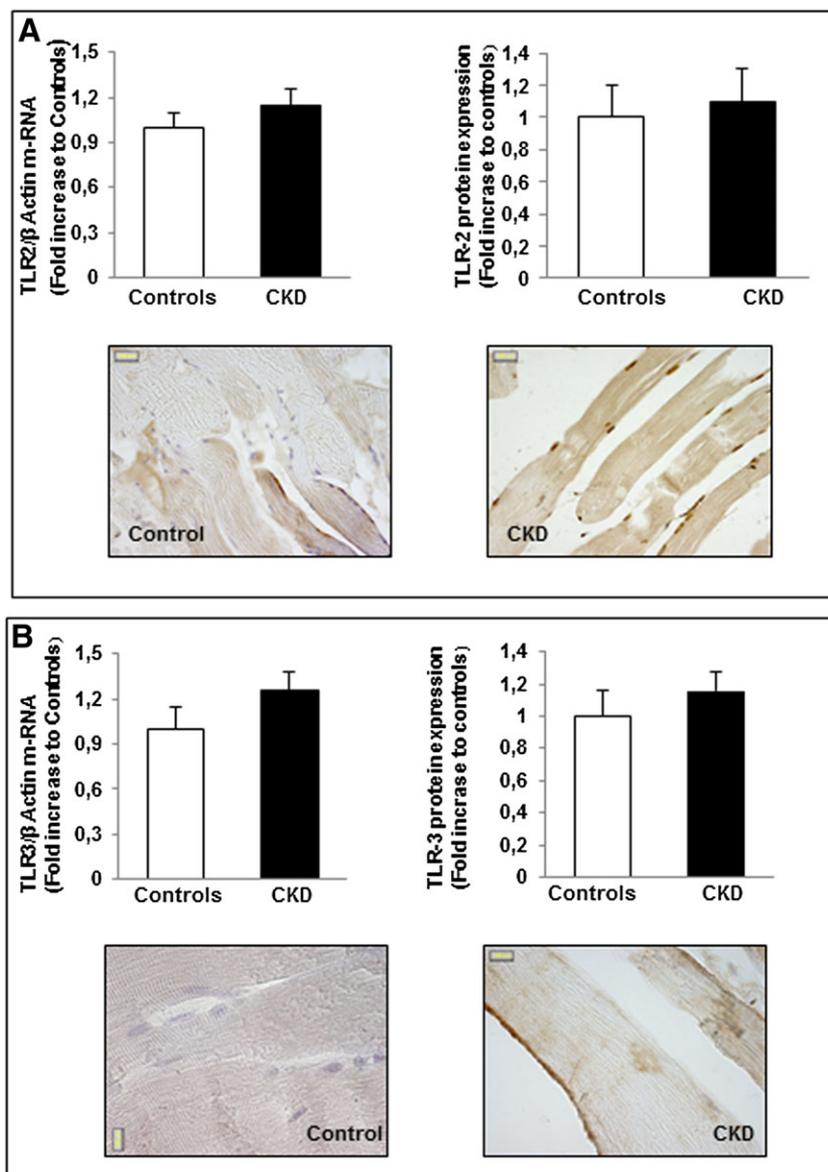
Figure 2 Expression of TLR4 mRNA (a) and protein (b–d) in normal skeletal muscle (*n* = 14) and in patients chronic kidney disease (CKD) (*n* = 29). TLR4 mRNA was evaluated by real-time PCR, and its protein by immunohistochemistry followed by image analysis (b, c) and western blot (d) of muscle lysates. Values are expressed as fold increase ± SEM to the control muscle. TLR4 mRNA was approximately two-folds increased vs. controls. TLR4 protein was absent or very faintly expressed in the normal muscle, while was overexpressed (by 1.5–3-folds) in CKD muscle (panel C). Western blots show up-regulated TLR4 in CKD with respect to controls (panel d). Blots were stripped and reprobed with anti β-actin antibody. The gel is representative of 12 CKD and 5 controls. CKD = chronic kidney disease. (Magnification: ×400–1000). The arrows indicate positive cells. *$P < 0.05$, **$P < 0.001$ vs. controls.
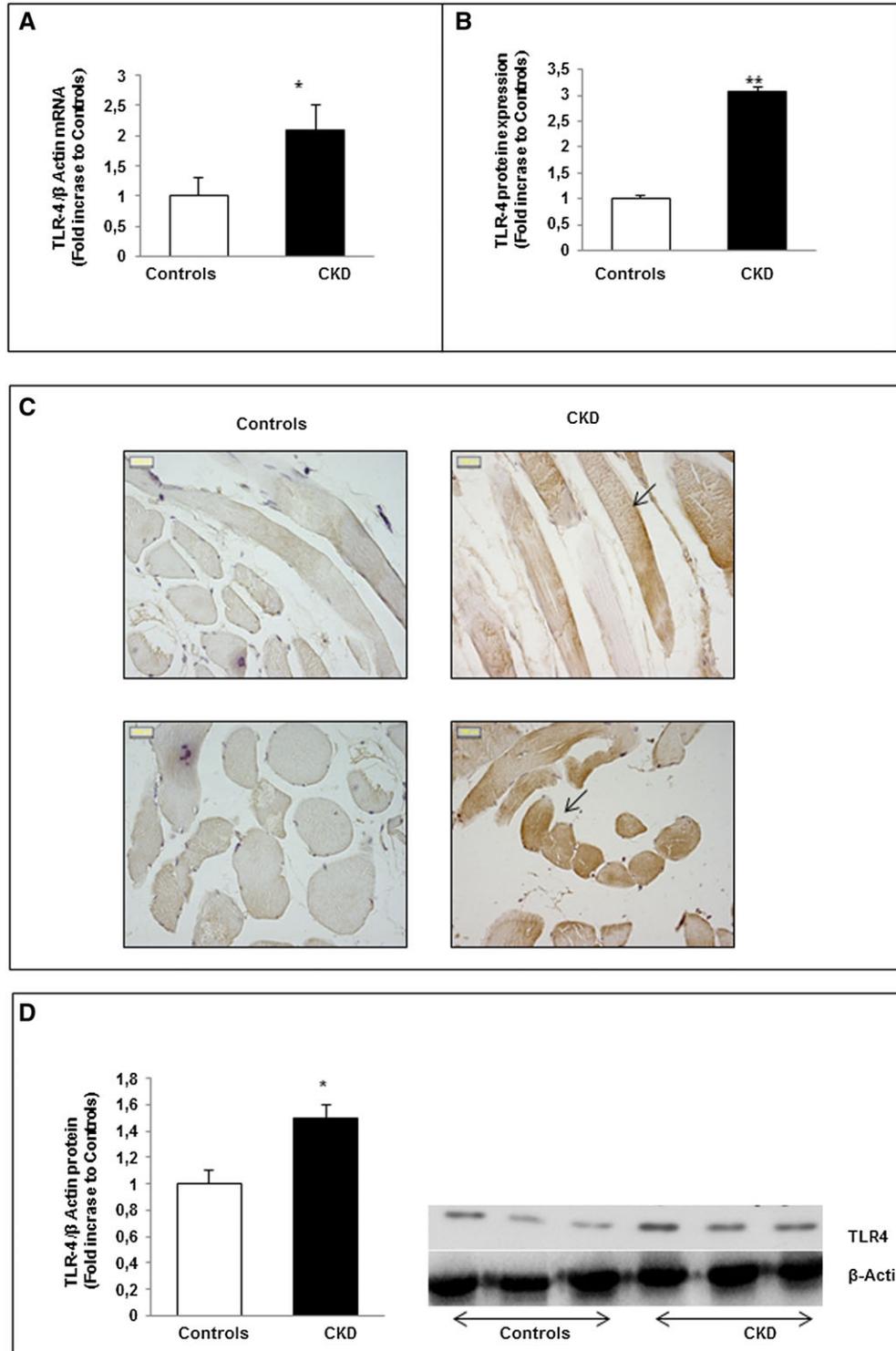
Figure 3 (Panel a). Phospho-p65 (P-p65) expression in the skeletal muscle of CKD patients and controls. Control ($n = 11$) muscle showed p-p65 positive nuclei in a very small percentage. This percentage increased significantly in patients with CKD ($n = 20$). The degree of positive nuclei was estimated by counting the number of p-p65 positive cells for 100 cells examined in average of five high-power fields. (Panel b) Phospho-IkB-α (p-IkB-α) expression in the skeletal muscle of CKD patients and controls. P-IkB-α was highly up-regulated in muscle of patients with CKD.(Panel c) Expression of TNF-α mRNA and protein in CKD ($n = 25$) and control ($n = 11$) muscle. TNF-α m-RNA was two-folds overexpressed in CKD samples with respect to the control tissue. The protein expression of TNF-α was minimally detectable in control samples, while it was markedly increased (by approximately five-folds) in muscle of CKD patients. (Panel d) Immunohistochemistry analysis for p-p38 in normal and CKD subjects. Staining was weakly diffused in normal tissue, but intensely expressed in uremia. (Panel e) Expression of Murf 1 and atrogin mRNA. Murf 1 and atrogin mRNAs expression level was determined by real time PCR. Both genes were over expressed in CKD muscle ($n = 12$) with respect to controls ($n = 10$). C = controls; CKD = chronic kidney disease. (Magnification: ×400–1000). The arrows indicate positive areas. Data are expressed as fold increase ± SEM to normal muscle. *$P < 0.05$ vs. C; **$P < 0.01$ vs. C.
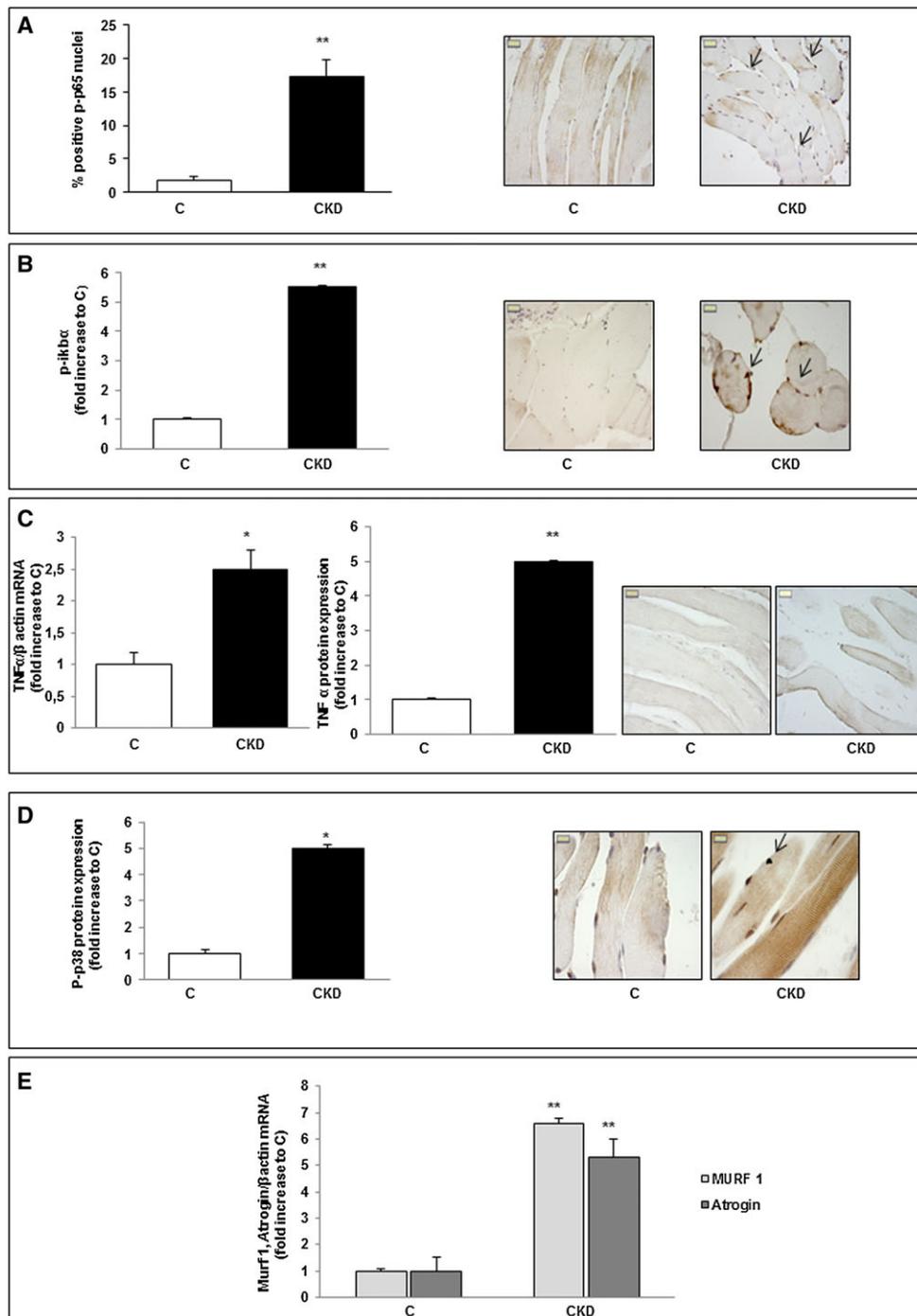
a first step we measured the abundance of NF-kB p65 (phosphorylated p65 subunit) (p-p65) and IkB-α (p-IkB-α) in muscle biopsies. Normal muscle showed p-p65 positive nuclear staining in a very small percentage (1.7±0.6%) of muscle fibres (Figure 3A). This percentage significantly rose to 17.4±2.5% in patients with CKD (Figure 3A). Also p-IkB-α and TNF-α expressions were increased in samples from CKD patients (Figure 3B and 3C, respectively).

Next, we evaluated the expression and localization of p38 as second messengers for TNF-α in uremia. Phosphorylated p38 (p38p) expression was present in the normal muscle at minimal levels. Conversely, p38p was up-regulated in CKD patients (Figure 3D).

To understand if different muscle fat infiltration could explain difference in cytokine expression in CKD, we studied Oil-red-O expression (see methods) in muscle biopsies of 10 CKD and 7 control subjects. Muscle fat infiltration was low and similar in CKD patients and control subjects (mean score 0.012±0.01 and 0.014±0.02, P=NS, respectively, in CKD patients and controls).

Finally, we studied MuRF-1, a muscle-restricted ubiquitin ligase involved in the accelerated protein degradation during various kinds of muscle atrophy and also depending on the TNF-α/p38 pathway.[29,30] MuRF-1 was highly up-regulated in muscles from CKD subjects. In addition also atrogin-1, another muscle-specific ubiquitin ligase triggering muscle atrophy, was up-regulated (Figure 3E). Collectively, these results indicate that CKD subjects have increased TLR4 expression/content and TLR4-driven signalling.

Clinical determinants of Toll-like receptor 4 expression in muscle

As a next step, we studied whether TLR4 expression in muscle can be predicted by clinical findings. Table 4 shows the associations between individual clinical data and logTLR4 content in skeletal muscle. LogTLR4 content in muscle was inversely related to eGFR, SGA, and haemoglobin levels, while it was directly related to cholesterol levels. Only as a trend ($P < 0.06$), muscle LogTLR4 content was related to serum CRP. There was no relationship between muscle logTLR4 and age, weight, fat and fat-free mass, cross-sectional muscle area, nPNA, estimated calorie intake, serum albumin, BUN, glucose, bicarbonate, triglycerides, phosphate, fibrinogen, and iron levels. Using the studied variables for inclusion into multivariate analysis models revealed SGA and eGFR only to contribute individually and significantly to the prediction of TLR4 expression in skeletal muscle ($R^2 = 0.53$, $P < 0.001$).

Uremic serum induces Toll-like receptor 4 expression, p38, and AKT activation in C2C12 myotubes

To explore whether circulating factors progressively retained in blood in uremia directly stimulate TLR4 and its driven signalling

Table 4 Univariate and multivariate analysis of the correlation between LogTLR4 protein expression and clinical characteristics in patients with CKD (n = 29)

| Clinical characteristics	Univariate		Multivariate	
	r	P	t	P
			(model r^2 = 0.53; P = 0.001)	
Age (years)	−0.043	NS		
Body weight (kg)	−0.310	NS		
Fat mass (kg)	−0.259	NS		
Fat-free mass (kg)	−0.339	NS		
BMI (kg/m²)	0.022	NS		
SGA	−0.49	0.01	4.205	0.01
Estimated GFR (mL/min.1.73 m²)	−0.44	0.03	3.902	0.03
BUN (mg/dL)	0.025	NS		
nPNA (g/kg)	−0.232	NS		
Serum albumin (g/dL)	0.220	NS		
LogCRP (mg/L)	0.354	0.06		
Haemoglobin (g/dL)	−0.380	0.05	0.039	NS
Cholesterol (mg/dL)	0.398	0.05	1.073	NS
Triglycerides (mg/dL)	0.102	NS		
Bicarbonate (mmol/L)	−0.181	NS		
Urate (mg/dL)	0.043	NS		
Calcium (mg/dL)	0.125	NS		
Phosphate (mg/dL)	0.037	NS		
PTH (pg/mL)	0.145	NS		
Fibrinogen (mg/dL)	−0.123	NS		
Glucose (mg/dL)	0.180	NS		
Iron (mg/dL)	0.220	NS		

Abbreviations: BMI, body mass index; CRP, C-reactive protein; GFR, glomerular filtration rate; nPNA, normalized nitrogen protein appearance; SGA, subjective global assessment.

in muscle, we treated C2C12 myotubes with 10% uremic serum for 0–6 h. Uremic serum induced a four-fold increase in TLR4 mRNA after 2 h, an effect which persisted until 6 h. Also TLR4 protein was up-regulated at 6 h respect to cells exposed to 10% normal serum (Figure 4A). In addition, uremic serum induced TNF-α mRNA expression (Figure 4B). Then, we investigated the effects of uremic serum on AKT and p38 phosphorylation. When C2C12 were exposed to uremic serum for 24 h, AKT phosphorylation was reduced by 40% with respect to cells treated with normal serum (Figure 4C). Moreover, uremic serum up-regulated p38 MAPK phosphorylation within 10 min (Figure 4D), an effect that persisted for 240 min.

P38 mitogen-activated protein kinase/protein kinase C is involved in uremic serum-induced Toll-like receptor 4 expression

To further investigate the role of p38 MAPK phosphorylation on uremic serum-induced TLR4 expression, we examined the role of p38 and its related signal pathway including protein kinase C (PKC), by the use of p38 MAPK and PKC inhibitors. Pretreatment of C2C12 myotubes with the p38 inhibitor SB203580 (10 μM) or the PKC inhibitors chelerythrine (5 μM), or staurosporine (0.2–0.4 μM), 1 h before serum exposure resulted in a marked

Figure 4 (Panel a). The effect of normal serum (NS) and uremic serum (US) on TLR4 mRNA and protein in C2C12 myotubes. Cells were incubated with 10% serum for 6 h. TLR4 mRNA expression was determined by real time PCR at different times and protein by western blot after six hours. (Panel b) The effect of uremic serum (US) on TNF-α gene expression in C2C12 myotubes. TNF-α mRNA expression was determined by real time PCR after 5 h treatment. (Panel c) Down-regulation of pAkt in uremic serum (US)-treated cells. pAkt was evaluated by western blot analysis after 24 h exposition to normal serum (NS) or US. Blots were stripped and reprobed with anti-pAkt antibody.(Panel d) Uremic serum (US) induces p-p38 during time course (0–240 min) in C2C12 myotubes. Blots were stripped and reprobed with anti β actin antibody. All results represent means ± SEM obtained from five independent experiments.*$P < 0.05$ vs. T0; **$P < 0.01$ vs. T0; §$P < 0.001$ vs. T0 and NS; +$P < 0.01$ vs. T0 and NS. NS = normal serum; US = uremic serum; pAkt = phospho-Akt; p-p38 = phospho-p38.
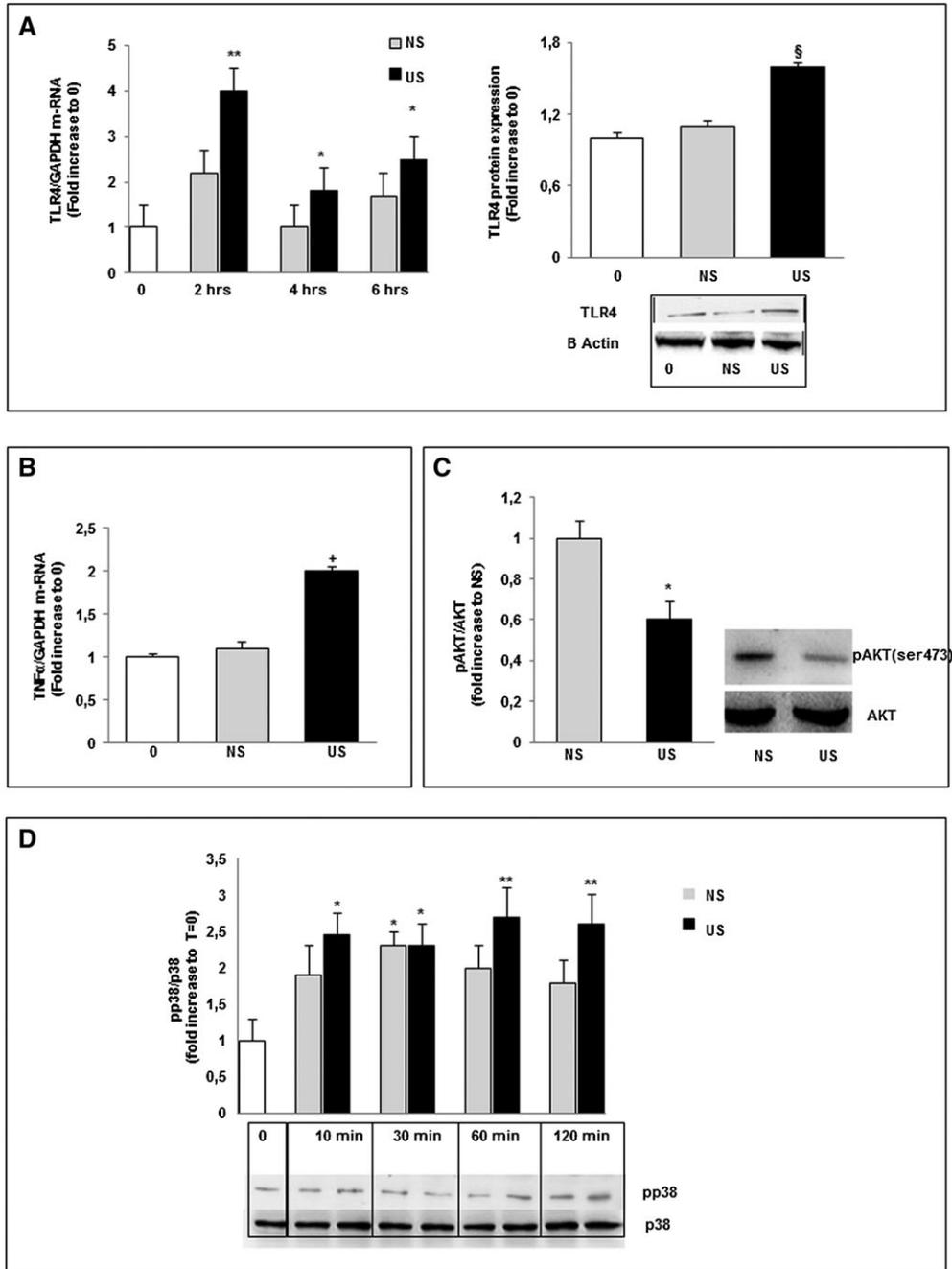
decrease in the serum induced TLR4 mRNA overexpression (Figure 5). These findings show the implication of the p38MAPK/PKC pathway in TLR4 activation by uremia.

Toll-like receptor 4 mediates the effect of uremic serum on Akt signalling

To evaluate whether the effects of uremic serum on the activation of TLR4 can be prevented by inhibiting TLR4 signalling, we pre-incubated the myotubes with VIPER, a specific TLR4 inhibitor, which acts by directly targeting the TLR4 adaptors Mal and TRAM, thus inhibiting TLR4-mediated responses.[31] As shown in Figure 6A, preincubation of myotubes with VIPER prevented the ability of uremic serum to up-regulate TNF-α.

As a next step, we employed gene silencing as an independent, albeit complementary method to examine the role of uremic serum on TLR4 regulation in muscle. TLR4 siRNA decreased TLR4 mRNA and protein in C2C12 myotubes (Figure 6B). TLR4 gene silencing reduced uremic serum-induced TNF-α (Figure 6C) and recovered AKT signalling (Figure 6D).

Discussion

In this study we tested the hypothesis that a TLR-driven inflammatory response occurs in skeletal muscle of patients with CKD. We identified the overexpression of TLR4 in muscle biopsies of stage 5 CKD patients and observed that this response was associated with NF-kB activation and the enrichment of multiple members of the NF-kB dependent inflammatory pathway. NF-kB is a well-recognized downward TLR4 transcription factor that directs the production of TNF-α and pro-inflammatory cytokines which are major mediators of protein breakdown and atrophy in the skeletal muscle.[32,33]

We have previously observed that pAkt is down-regulated in skeletal muscle of CKD subjects.[12] Also in accordance with one previous observation,[9] the TNF-alpha gene, that is highly regulated by NFkB,[12,13] was overexpressed in muscle from CKD subjects.TNF-α is also by itself an activator of NF-kB, thus leading to a positive feedback loop which potentiates muscle abnormalities.[32] In addition, TNF-α activates mitogen-activated protein kinases (MAPKs), including p38 and JNK[33] to increase protein breakdown by atrogin1/MAFbx gene expression in skeletal muscle.[34] We previously observed up-regulated JNK in muscle of CKD patients.[12] In addition, we observed that p38 MAPK, a second messenger for TNF-α,[35–37] is up-regulated in uremic muscle. The p38 MAPK activity in skeletal muscle is regulated by several catabolic conditions, such as ageing,[38] type 2 diabetes,[39] limb immobilization,[40] and neurogenic atrophy,[41] suggesting that the TNF-α/p38 MAPK driven signalling takes place as a common mechanism in different catabolic conditions.

According to previous observations[1,12] muscle pAkt is down-regulated in uremia. In muscle physiology, the activation of the Akt signalling pathway mediates the balance

Figure 5 Effects of p38 inhibitor (10 μM SB203580), and pKC inhibitors (0.2–4 μM staurosporine or 10 μM chelerythrine) on uremic serum (US)-induced TLR4 mRNA. To further investigate the mechanism of uremic serum-induced TLR4 expression, we examined the role of p38 and its related signal pathway including protein kinase C (PKC). Pretreatment of C2C12 myotubes with the p38 inhibitor SB203580 (10 μM) and the PKC inhibitors chelerythrine (5 μM) and staurosporine (0.2–0.4 μM) 1 h before serum exposure, resulted in a marked decrease in the serum-induced TLR4 mRNA overexpression. All results represent means ± SEM obtained from three independent experiments.*P < 0.05 vs. T0;.*P < 0.05 vs. US; **P < 0.01 vs. US. US = uremic serum; che = chelerythrine; stau = staurosporine.
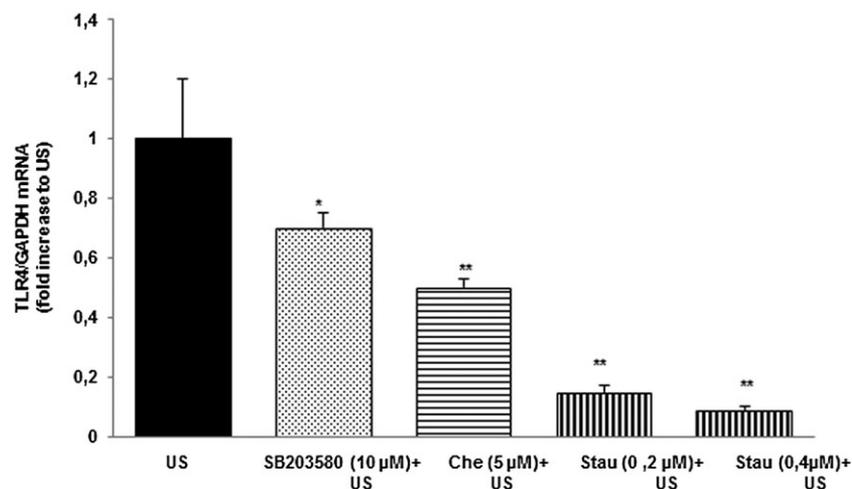
Figure 6 Effect of uremic serum (US) on TNF-α gene expression and pAkt in C2C12 myotubes treated with TLR4 inhibitors or silenced for TLR4. (Panel a) Preincubation of myotubes with VIPER, a specific TLR4 inhibitor, prevented the ability of US to up-regulate TNF-α. (Panel b) As a next step, we employed gene silencing as an independent method to examine the role of US on TLR4 regulation in muscle. C2C12 were transfected with 60 nM siRNA NC and TLR4-specific siRNA and the respective mRNA and protein were evaluated after 24 h. TLR4 siRNA decreased TLR4 mRNA and protein in C2C12 myotubes. (Panel c) Effect of US on TNF-α mRNA in C2C12 silenced for TLR4. TLR4 gene silencing blunted US-regulated TNF-α. (Panel d) Effect of US on Akt phosphorylation. C2C12 with no knockdown and transfected with TLR4 siRNA were exposed for 24 hours to NS or US. pAkt was evaluated by western blot and membrane was stripped and reprobed with anti Akt antibody. TLR4 knockdown restored pAkt signalling. All results represent means ± SEM obtained from three independent experiments. $\#P < 0.01$ vs. NC; $\S P < 0.01$ vs. NC + US; $°P < 0.05$ vs. US; $*P < 0.001$ vs. US and CP + US. NS = normal serum; US = uremic serum; pAkt = phospho-Akt; NC = non-specific negative control siRNA; VIPER = viral inhibitory peptide of TLR4; CP7 = inert control peptide.
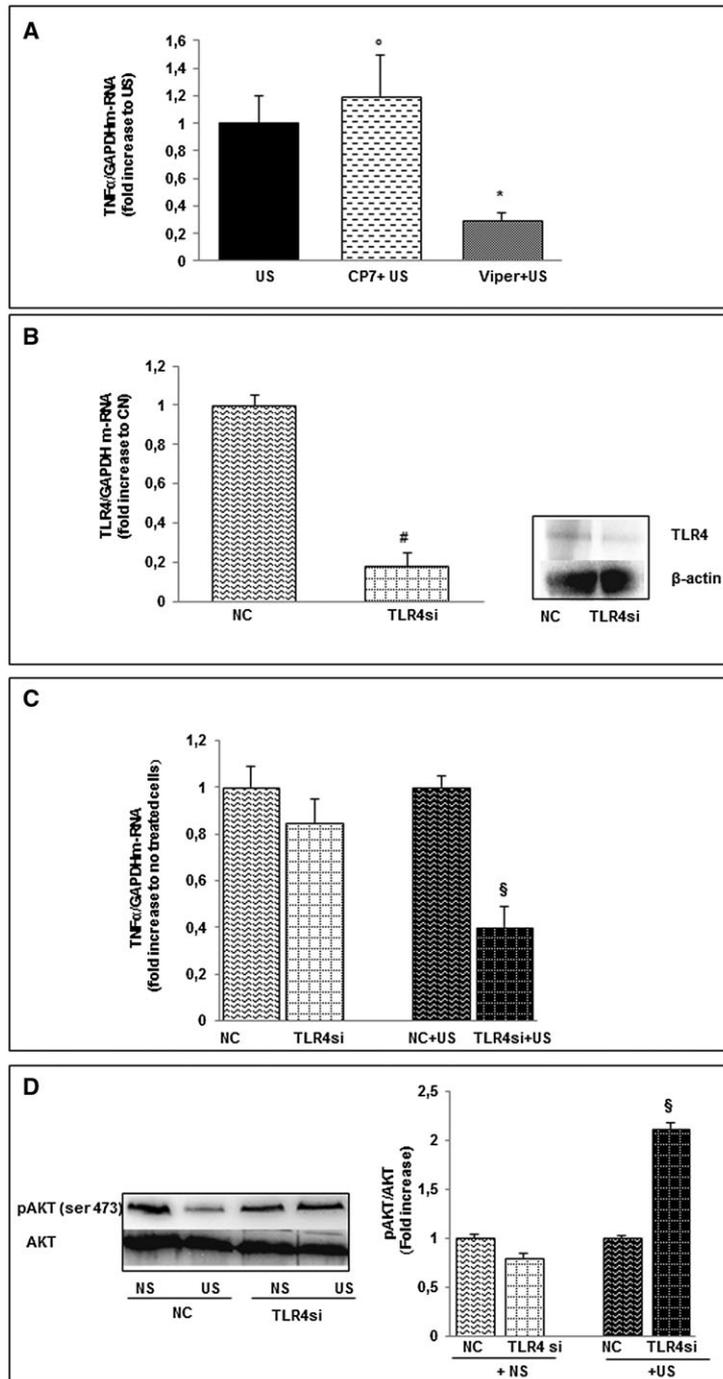
between the anabolic and catabolic reactions. Inhibition of this pathway up-regulates, while its activation down-regulates, the expression of the transcription factors MAFbx and MuRF-1 via inhibition of the Foxo family of transcription factors.[19,33] During atrophy, MuRF-1 and MAFbx direct the polyubiquitination of proteins to target them for proteolysis by the 26S proteasome, mediating muscle break-down.[37] As a signature response of decreased pAkt, we observed also an overexpression of the regulators of protein degradation atrogin-1/MAFbx and MuRF1 in muscle from CKD patients.

Collectively, these results indicate that Stage 5 CKD subjects have increased TLR4 expression/content and TLR4-driven signalling in muscle. To better understand the significance of our data, we studied the associations between TLR4 content in muscle and several clinical findings. We observed that both low SGA score (which indicates an overall concept of impaired nutrition) and eGFR (an index of residual renal function) play an independent role to predict TLR4 content in muscle of CKD patients. Thus, according to our findings, both wasting and progressive loss of residual renal function are predictive of muscle TLR4. The SGA has been validated as reliable tool to assess protein-energy wasting. A single SGA assessment has been shown to be associated with morbidity, hospitalization, and risk of short-term mortality in dialysis patients. In our study TLR4 content in muscle rose progressively along with progressive decline of residual renal function, with a two-fold increase in TLR4 as eGFR declined from 13 to 4 mL/min. However, although TLR4 was associated with SGA, we could not observe an association between TLR4 and fat free mass in patients with CKD. This finding suggests that TLR4 up-regulation is a part of the stress response that takes place in overt uremia. In contrast, metabolic acidosis causes a more precocious (Stage 4) disturbance of muscle protein metabolism in patients with CKD.[42] According to our findings, in patients with CKD metabolic acidosis and up-regulation of native immunity are, respectively, part of the early and late muscle stress response.

It is tempting to speculate that in Stage 5 CKD patients the decline of the residual renal function causes the retention of still unidentified circulating compound(s) which accumulate in uremia and is sensed by TLRs in muscle. To examine whether uremic milieu directly activates TLR4 signalling in human muscle, we exposed differentiated C2C12 mice myotubes to uremic serum. Interestingly, uremic serum rapidly stimulated the mRNA expression of TLR4 and TNF-α, and depressed pAkt. Moreover, the gene expression of TNF-α and pAkt down-regulation activated by uremic serum were prevented in skeletal muscle cells by selective TLR4 inhibition or TLR4 knockdown. We conclude that the regulation of TNF-α in myotubes by uremic serum is mediated by TLR4 expression and signalling. Overall, our data suggest that the up-regulated

TLR4 response in muscle of CKD patients translate the metabolic alterations of uremia into decreased insulin signal and atrophy.

What sensing and signalling mechanisms are involved in TLR4-dependent immune activation in uremic muscle? Besides endotoxin,[43] a variety of putative endogenous TLR ligands have been described in the literature, including heat shock proteins that bind TLR4,[44] the small ribonuclear RNA U1 that binds TLR3,[45] and various extracellular matrix and membrane components.[46] The circulating molecules which can be sensed by skeletal muscle in uremia to mount a TLR4 response are unknown. One possibility is that alterations in calcium and/or phosphate metabolism, which increase the risk for vascular calcification, may act as a yet another nidus for a local inflammatory response.[47] However in patients studied here, TLR4 expression in muscle was not associated with serum phosphate, calcium, or PTH levels. Another possibility is that some molecule are sensed by muscle as danger signal. In patients with diabetes, TLRs sense high glucose, high free fatty acids, AGEs, or other molecules,[48,49] as danger signals and subsequently mediate the inflammatory cascade to promote progression of diabetic complications.[49] However, patients studied here had nondiabetic CKD. A number of uremic retention solutes, such as guanidine compounds, exert pro- as well as anti-inflammatory effects on monocyte/macrophage function.[50] In addition, in recent years the gut microbiota has been linked to this pathogenesis of inflammation in uremia.[51] Specifically, lipopolysaccharide (LPS or endotoxin), an outer membrane component of Gram negative bacteria, is considered to be a causative factor for insulin resistance.[51] It has been postulated that uremia alter gut flora growth and intestinal wall permeability, elevating enterobacterial production and translocation of LPS into the systemic circulation.[51] In patients studied here the level of circulating endotoxins was borderline high, suggesting that circulating endotoxins might be responsible for the changes in TLR4 muscle expression and inflammatory changes. Clearly, this should be addressed in future studies.

In summary, the present results provide compelling evidence that in uremia the activation of TLR4 present in skeletal muscle cells triggers an innate immune response that mediates a down-regulation of pAkt (anabolic signal) and up-regulates a TNF-α dependent inflammatory pathway. The identity of the ligand that activates TLR4 in this setting remains uncertain.

Our data also suggest that patients with CKD have increased muscle capacity to mount a rapid and possibly uncontrolled inflammatory response in the presence of TLR4 ligands, such as during an acute illness or infection. Targeting TLR4 may prove to be a therapeutic modality, improving insulin signalling and preventing the profound catabolic consequences of insulin resistance in CKD.

Acknowledgements

Conflict of Interest

Giacomo Garibotto has received research grants and speaker honorarium from Fresenius-Kabi (Bad Homburg, Germany). Daniela Verzola, Alice Bonanni, Antonella Sofia, Francesco Montecucco, Elena D'Amato, Valeria Cademartori, Emanuele Luigi Parodi, Francesca Viazzi, Chiara Venturelli, and Giuliano Brunori have declared that they have no conflict of interest relevant to this work.

References

1. Mitch WE. Proteolytic mechanisms, not malnutrition, cause loss of muscle mass in kidney failure. *J Ren Nutr* 2006;**16**:208–211.
2. Vaziri ND, Pahl MV, Crum A, et al. Effect of uremia on structure and function of immune system. *J Ren Nutr* 2012;**22**:149–156.
3. Anders HJ, Andersen K, Stecher B. The intestinal microbiota, a leaky gut, and abnormal immunity in kidney disease. *Kidney Int* 2013;**83**:1010–1016.
4. Onesti JK, Guttridge DC. Inflammation based regulation of cancer cachexia. *Biomed Res Int* 2014;**168**.
5. Egerman MA, Glass DJ. Signaling pathways controlling skeletal muscle mass. *Crit Rev Biochem Mol Biol* 2014;**49**:59–68.
6. Bergström J, Lindholm B. Malnutrition, cardiac disease, and mortality: an integrated point of view. *Am J Kidney Dis* 1998;**32**:834–841.
7. Kaysen GA. Progressive inflammation and wasting in patients with ESRD. *Clin J Am Soc Nephrol* 2014;**9**:225–226.
8. Zhang L, Rajan V, Lin E, et al. Pharmacological inhibition of myostatin suppresses systemic inflammation and muscle atrophy in mice with chronic kidney disease. *FASEB J* 2011;**25**:1653–1663.
9. Zhang L, Pan J, Dong Y, et al. Stat3 activation links a C/EBPδ to myostatin pathway to stimulate loss of muscle mass. *Cell Metab* 2013;**18**:368–379.
10. Garibotto G, Sofia A, Procopio V, et al. Peripheral tissue release of interleukin-6 in patients with chronic kidney diseases: effects of end-stage renal disease and microinflammatory state. *Kidney Int* 2006;**70**:384–390.
11. Shah VO, Dominic EA, Moseley P, et al. Hemodialysis modulates gene expression profile in skeletal muscle. *Am J Kidney Dis* 2006;**48**:616–628.
12. Verzola D, Procopio V, Sofia A, et al. Apoptosis and myostatin mRNA are upregulated in the skeletal muscle of patients with chronic kidney disease. *Kidney Int* 2011;**79**:773–782.
13. Argiles JM, Busquet M, Lopez-Soriano FJ. The pivotal role of cytokines in muscle wasting during cancer. *Int J Biochem Cell Biol* 2005;**37**:2036–2046.
14. Goodman MN. Tumor necrosis factor induces skeletal muscle protein breakdown in rats. *Am J Physiol Endocrinol Metab* 1991;**260**:E727–E730.
15. Haddad F, Zaldivar F, Cooper DM, et al. IL-6-induced skeletal muscle atrophy. *J Appl Physiol* 2005;**98**:911–917.
16. Thomas SS, Dong Y, Zhang L, et al. Signal regulatory protein-α interacts with the insulin receptor contributing to muscle wasting in chronic kidney disease. *Kidney Int* 2013;**84**:308–316.
17. Chen Y, Biada J, Sood S, Rabkin R. Uremia attenuates growth hormone-stimulated insulin-like growth factor-1 expression, a process worsened by inflammation. *Kidney Int* 2010;**78**:89–95.
18. Garibotto G, Russo R, Sofia A, et al. Effects of uremia and inflammation on growth hormone resistance in patients with chronic kidney disease. *Kidney Int* 2008;**74**:937–945.
19. Bailey JL, Zheng B, Hu Z, et al. Chronic kidney disease causes defects in signalling through the insulin receptor substrate/phosphatidylinositol 3-kinase/Akt pathway: implications for muscle atrophy. *J Am Soc Nephrol* 2006;**17**:1388–1394.
20. Miyamoto T, Carrero JJ, Qureshi AR, et al. Circulating follistatin in patients with chronic kidney disease: implications for muscle strength, bone mineral density, inflammation, and survival. *Clin J Am Soc Nephrol* 2011;**6**:1001–1008.
21. Wang XH, Mitch WE. Mechanisms of muscle wasting in chronic kidney disease. *Nat Rev Nephrol* 2014;**10**:504–516.
22. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010;**11**:373–384.
23. Takeda K, Akira S. Regulation of innate immune responses by Toll-like receptors. *Jpn J Infect Dis* 2001;**54**:209–219.
24. Lang CH, Silvis C, Deshpande N, et al. Endotoxin stimulates in vivo expression of inflammatory cytokines tumor necrosis factor alpha, interleukin-1beta, -6, and high-mobility-group protein-1 in skeletal muscle. *Shock* 2003;**19**:538–546.
25. Frost RA, Lang CH. Regulation of muscle growth by pathogen-associated molecule. *J Anim Sci* 2008;**86**:E84–E93.
26. Anders HJ, Schlöndorff D. Toll-like receptors: emerging concepts in kidney disease. *Curr Opin Nephrol Hypertens* 2007;**16**:177–183.
27. Anders HJ. Toll-like receptors and danger signaling in kidney injury. *J Am Soc Nephrol* 2010;**21**:1270–1274.
28. Levey AS, Stevens LA. Estimating GFR using the CKD epidemiology collaboration (CKD-EPI) creatinine equation: more accurate GFR estimates, lower CKD prevalence estimates, and better risk predictions. *Am J Kidney Dis* 2010;**55**:622–627.
29. Visser R, Dekker FW, Boeschoten EW, et al. Reliability of the 7-point subjective global assessment scale in assessing nutritional status of dialysis patients. *Adv Perit Dial* 1999;**15**:222–225.
30. de Mutsert R, Grootendorst DC, Boeschoten EW, et al. Subjective global assessment of nutritional status is strongly associated with mortality in chronic dialysis patients. *Am J Clin Nutr* 2009;**89**:787–793.
31. Paigen B, Morrow A, Holmes PA, et al. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 1987;**68**:231–240.
32. Garg AK, Aggarwal BB. Reactive oxygen intermediates in TNF signaling. *Mol Immunol* 2002;**39**:509–517.
33. Schakman O, Dehoux M, Bouchuari S, et al. Role of IGF-I and the TNFα/NF-kB pathway in the induction of muscle atrogenes by acute inflammation. *Am J Physiol Endocrinol Metab* 2012;**303**:E729–E739.
34. Bodine SC, Latres E, Baumhueter S, et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 2001;**294**:1704–1708.
35. Lysakova-Devine T, Keogh B, Harrington B, et al. Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46 specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule. *J Immunol* 2010;**185**:4261–4271.
36. Kumar A, Takada Y, Boriek AM, et al. Nuclear factor-kB: its role in health and disease. *J Mol Med* 2004;**82**:434–448.
37. Ping Li Y, Chen Y, John J, et al. TNF-α acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB J* 2005;**19**:362–370.
38. Williamson D, Gallagher P, Harber M, et al. Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and

acute exercise on human skeletal muscle. *J Physiol* 2003;**547**:977–987.

39. Koistinen HA, Chibalin AV, Zierath JR. Aberrant p38 mitogen-activated protein kinase signaling in skeletal muscle from Type 2 diabetic patients. *Diabetologia* 2003;**46**:1324–1328.

40. Childs TE, Spangenburg EE, Vyas DR, et al. Temporal alterations in protein signaling cascades during recovery from muscle atrophy. *Am J Physiol End Metab* 2003;**285**: C391–C398.

41. Di Giovanni S, Molon A, Broccolini A, et al. Constitutive activation of MAPK cascade in acute quadriplegic myopathy. *Ann Neurol* 2004;**55**:195–206.

42. Garibotto G, Russo R, Sofia A, et al. Skeletal muscle protein synthesis and degradation in patients with chronic renal failure. *Kidney Intern* 1994;**45**:1432–1439.

43. Doyle A, Zhang G, Abdel Fattah EA, et al. Toll-like receptor 4 mediates lipopolysaccharide-induced muscle catabolism via coordinate activation of ubiquitin–proteasome and autophagy–lysosome pathways. *FASEB J* 2011;**25**:99–110.

44. Ohashi K, Burkart V, Flohé S, et al. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol* 2000;**164**: 558–561.

45. Hoffman RW, Gazitt T, Foecking MF, et al. U1 RNA induces innate immunity signaling. *Arthritis Rheum* 2004;**50**:2891–2896.

46. Cohen S, Brault JJ, Gygi SP, et al. During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation. *J Cell Biol* 2009;**185**:1083–1095.

47. Doherty TM, Asotra K, Fitzpatrick LA. Calcification in atherosclerosis: bone biology and chronic inflammation at the arterial crossroads. *Proc Natl Acad Sci U S A* 2003;**100**:11201–1120.

48. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 2006;**6**:823–835.

49. Verzola D, Cappuccino L, D'Amato E, et al. Enhanced glomerular Toll-like receptor 4 expression and signaling in patients with type 2 diabetic nephropathy and microalbuminuria. *Kidney Int* 2014;**86**: 1229–1243.

50. Glorieux GL, Dhondt AW, Jacobs P, et al. In vitro study of the potential role of guanidines in uremia-related atherogenesis and infection. *Kidney Int* 2004;**65**:2184–2192.

51. Ramezani A, Raj DS. The gut microbiome, kidney disease, and targeted interventions. *J Am Soc Nephrol* 2014;**25**:657–670.

52. von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2015. *J Cachexia Sarcopenia Muscle* 2015;**6**:315–316.