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Toll-like receptor expression in monocytes in patients with chronic kidney disease and haemodialysis: relation with inflammation

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Abstract

Background. Inflammation is one of the main contributors to atherosclerosis in haemodialysis (HD) patients. Activation of Toll-like receptors (TLRs) leads to inflammatory response. In this study, we aimed to evaluate the expression of TLRs on monocytes and relate their expression with inflammation in chronic kidney disease (CKD) and HD patients.

Methods. Thirty-four age- and gender-matched controls and stage 3–4 CKD patients and thirty-two HD patients were included in each study group. The effect of HD on the expression of Toll-like receptor-2 (TLR-2) and Toll-like receptor-4 (TLR-4) on $CD14^+$ monocytes was determined at the beginning (baseline), during (120 min) and following (300 min and 24 h) HD and compared with control and stage 3–4 CKD groups. The HD procedure was performed by using low-flux polysulphone dialysers. In addition, serum IL-6 levels were evaluated in both groups at baseline and after a HD session.

Results. The percentage of $CD14^+$ monocytes expressing TLR-2 were similar in all of the study groups, whereas the percentage of $CD14^+$ monocytes expressing TLR-4 were significantly lower in both stage 3–4 CKD and HD patients at baseline than in controls. The mean fluorescence intensities (MFI) of TLR-2 were significantly lower in controls than in stage 3–4 CKD and HD patients at baseline. The MFI of TLR-4 was similar in all of the groups. The percentage of $CD14^+$ monocytes expressing TLR-2 did not change during and after HD. The MFI of TLR-2 decreased at 120 min of HD compared with baseline (1837 ± 672 vs 1650 ± 578, $P < 0.05$), and recovered back to baseline values at 300 min and at 24 h post-HD. MFI of TLR-4 increased at 24 h compared with baseline (941 ± 294 vs 1087 ± 441, $P < 0.05$). Serum IL-6 levels correlated with MFI of TLR-2 and TLR-4 in stage 3–4 CKD patients and in HD patients at baseline and after HD in univariate analysis. Stepwise multiple regression analysis revealed that MFI of TLR-2 was an independent determinant of serum IL-6 concentrations in stage 3–4 CKD and in HD patients at baseline, at 300 min and at 24 h post-HD.

Conclusions. Our study demonstrates that TLR-2 is associated with the inflammatory response of non-dialysed and dialysed CKD patients.

Keywords: chronic kidney disease; haemodialysis; inflammation; Toll-like receptor

Introduction

An increased level of inflammatory markers is associated with increased cardiovascular mortality in haemodialysis (HD) patients [1]. Loss of renal function, infection of vascular access, bio-incompatibility of dialysis membranes and diffusion of endotoxins from non-sterile dialysate to the blood are among the factors contributing to increased inflammation in HD patients [2]. Dialysis membranes and dialysate have been shown to stimulate the secretion of inflammatory markers such as IL-1β from monocytes *in vitro* and C-reactive protein (CRP) and IL-6 *in vivo* through mechanisms such as complement activation [3].

Toll-like receptor-2 (TLR-2) and Toll-like receptor-4 (TLR-4) are involved in innate immunity. Peptidoglycans activate TLR-2, and lipopolysaccharide (LPS) activates TLR-4 [4,5]. A ligand binding to these receptors activates the intracellular nuclear factor-κB (NF-κB) pathway and enhances the expression of NF-κB-controlled genes such as for inflammatory cytokines and adhesion molecules in monocytes, macrophages, dendritic cells and endothelial cells [4–6]. Activation of these receptors leads to systemic inflammation in the host [6]. Data about the effect of uraemia and HD on TLR-2 and TLR-4 expression on monocytes are limited. Compared with healthy controls, expression of TLR-2 and TLR-4 on monocytes was similar or lower in haemodialysis and pre-dialysis chronic kidney disease (CKD) patients, respectively [7,8]. To the best of our knowledge, evolution during haemodialysis or relation to inflammatory status was never evaluated in a CKD population.

In this study, we hypothesized that uraemia and HD procedure may affect the expression of TLR-2 and TLR-4 on monocytes and endothelial cells leading to inflammation in stage 3–4 CKD and HD patients. Therefore, we evaluated the expression of TLRs on $CD14^+$ monocytes and related their expression with inflammation in stage 3–4 CKD patients and HD patients before, during, and after completion of a HD session. Since interleukin-6 (IL-6) stimulates synthesis of CRP and predicts cardiovascular mortality better than any other markers of inflammation such as CRP, albumin and TNF-α in HD patients [9], we studied IL-6 as a marker of inflammation in this study.

Materials and methods

Study population

The study protocol was approved by the Institutional Review Board at the Marmara University, and written informed consent was obtained from all patients. Thirty-two HD patients undergoing chronic HD treatment for at least 3 months, stage 3–4 chronic kidney disease (CKD) patients and 34 healthy controls were included into the study. Exclusion criteria were: (i) signs or symptoms of clinical infection in the previous 3 months; (ii) glucocorticoid or non-steroidal anti-inflammatory medication use other than acetylsalicylic acid; (iii) central line insertion or any other invasive procedure within the previous month; (iv) HIV infection; (v) chronic hepatitis B or C infections; and (vi) history of neoplastic, inflammatory or immunological diseases.

HD procedures were performed using polysulphone low-flux dialysers (Fresenius Medical Care, Lexington, KY, USA). All HD patients underwent HD for 4 h by cannulation of the arteriovenous fistula, and using sterile bicarbonate concentrate, heparin sodium and reverse osmosis water. Dialysate and blood flow rates were 500 and 350 mL/min. By using a kinetic chromogenic limulus amebocyte lysate assay (Lonza, Walkersville, MD, USA) as previously described [9], all pre-dialysis dialysate LPS concentrations were <0.25 EU/mL on the day that patients underwent their evaluation [10]. The sensitivity of the assay is 0.005 EU/mL. In addition, a novel biological test method to assess the cytokine-induction capacity of dialysate samples, also indicating non-LPS pro-inflammatory elements such as peptidoglycans and deoxynucleotides, was performed, and again, no activity in dialysate samples was detected [10].

Collection of blood samples

In order to determine the time points of blood sampling, we first performed a preliminary study in eight HD patients (four female, mean age 48 ± 13 years and mean duration of HD 58 ± 59 months). In this first part, we measured the expression of TLR-2 and TLR-4 on $CD14^+$ monocytes at baseline (prior to HD treatment), at 15, 30, 60, 120 and 240 min (end of the session) of HD in blood collected from both inlet and outlet of filter (outlet sample collected 30 s after the inlet sample), and at 1 h (300 min) and 24 h after the end of the HD session from a peripheral vein. In these patients, leucocyte counts were performed using an automatic cell counter. Differential leucocyte counts were obtained by counting cells stained on smears of blood samples anti-coagulated with ethylenediamine tetra-acetic acid (EDTA) under the microscope. In the second part of the study, we included a group of healthy controls ($n = 34$), a group of stage 3–4 CKD patients ($n = 34$) and a group of HD patients ($n = 32$). Based on the results of the preliminary study, blood samples from HD patients were collected at the beginning of a mid-week routine dialysis session (baseline) and at 120 min into the HD session from their arterial lines. Blood samplings were repeated 1 h after the end of HD (300 min) and at 24 h from a peripheral vein. Fasting blood samples of controls and stage 3–4 CKD patients were drawn from a peripheral vein. TLR-2 and TLR-4 expression on $CD14^+$ monocytes were determined by flow cytometry in these EDTA-anti-coagulated blood samples. Plasma and serum samples were separated by immediate centrifugation at 4°C (1500 *g* for 10 min), aliquoted and stored at −80°C until analysis.

In a subpopulation of eight HD patients (two female, mean age 56 ± 17 years and mean duration of HD 72 ± 84 months), we further collected blood samples at baseline and at 15, 30, 60, 120 and 240 min (end of the session) of HD in blood collected from both inlet and outlet (30 s after the inlet). Blood samplings were also repeated 1 h after the end of HD (300 min) and at 24 h from a peripheral vein. In this population, we also determined the expression of TLRs on $CD14^+CD16^+$ monocytes by flow cytometry. Plasma and serum samples were separated by immediate centrifugation at 4°C (1500 *g* for 10 min), aliquoted and stored at −80°C for the determination of soluble TLR-2 (sTLR-2) levels.

Flow cytometric analysis

Flow cytometric analysis was performed in fresh samples within 15 min of blood collection as previously described [11]. In brief, 100 μL of EDTA-anti-coagulated blood was stained with fluorochrome-conjugated monoclonal antibodies (mAb): PE-labelled mAb for human TLR-4 (clone HTA125), and TLR-2 (clone TL2.1) and FITC-labelled mAb for CD14 (clone 61D3), APC-labelled mAb for CD16 (clone CB16) and their corresponding isotype controls (all from e-Bioscience, San Diego, CA, USA). After 20 min of pre-incubation with mAb in the dark, the samples were fixed (2% paraformaldehyde/PBS), and erythrocytes were lysed with lysing buffer (0.155 M ammonium chloride, 0.01 M potassium bicarbonate and 0.127 M EDTA). Cells were then washed twice in PBS solution and were re-suspended in PBS solution before analysis. Flow cytometry was performed by using a FACScan Analyser (Becton Dickinson, Mountain View, CA, USA). Monocytes were gated or identified based on their light scatter properties and their CD14 positivity [12]. Acquisition was stopped after 20 000 $CD14^+$ or $CD14^+CD16^+$ monocytes were acquired. Flow cytometric data were analysed by CellQuest 3.3 software (BD Biosciences, San Jose, CA, USA).

Determination of IL-6 and soluble TLR-2

Serum levels of IL-6 (BD Biosciences Pharmingen, San Diego, CA, USA) and plasma levels of sTLR-2 (R&D systems, catalogue no: DY2616) were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Serum IL-6 analyses were evaluated in

Table 1. Demographic and laboratory data in stage 3–4 CKD and HD patients

	Control (n = 34)	Stage 3–4 CKD (n = 34)	HD patients (n = 32)
Age (years, range)	54.1 ± 12.8	52.1 ± 13.5	49.8 ± 15.2
Gender (women/men)	17/17	13/21	17/15
Duration of haemodialysis (month)			69 ± 66
Causes of CKD, n (%)			
Diabetes mellitus		2 (5.9)	4 (12.5)
Hypertension		11 (32.3)	11 (34.4)
Glomerulonephritis		7 (20.6)	6 (18.8)
Cystic kidney disease		3 (8.8)	3 (9.4)
Other		6 (17.7)	4 (12.5)
Unknown		5 (14.7)	4 (12.5)
Use of erytropoietin, n (%)		4 (11.8)	27 (85.3)
Kt/V			1.42 ± 0.37
Creatinine (mg/dL)	0.8 ± 0.2	3.2 ± 1.5[†]	
Haemoglobin (g/dL)	14.1 ± 1.2	12.3 ± 1.8	11.1 ± 1.1[*,**]
Albumin (g/dL)	4.7 ± 0.4	4.4 ± 0.6 [≠]	4.0 ± 0.4 [*,†]
Calcium (mg/dL)	9.3 ± 0.5	9.3 ± 0.8	9.1 ± 0.9
Phosphorus (mg/dL)	3.4 ± 0.5	4.3 ± 1.0[‡]	5.7 ± 1.5[*,†]
Ca × P (mg^2/dL^2)	31 ± 6	40 ± 10[‡]	52 ± 14[*,†]
PTH (pg/mL)		192 ± 148	362 ± 369
Ferritin (ng/mL)		151 ± 136	646 ± 407*

Values are expressed as mean ± SD unless indicated differently. PTH, parathyroid hormone; Ca × P, calcium–phosphorus product.
[*]P < 0.0001 vs CKD group.
[†]P < 0.0001 vs control group.
[‡]P <0.05 vs control group.

CKD patients and at baseline, at 1 h after the end of HD session (300 min) and at 24 h in HD patients. sTLR-2 levels were determined in eight HD patients in plasma samples withdrawn from both inlet and outlet of the filter simultaneously at baseline and at 15, 30, 120, and 240 min of the HD session. The concentrations of IL-6 and sTLR-2 were calculated by reference to standard curves performed with the corresponding recombinant molecules. All samples were tested in duplicate. Serum concentrations (C) of IL-6 at 300 min and sTLR-2 at outlet were corrected for volume contraction based on plasma protein concentrations according to the formula: C corrected/C post = P pre/P post, where P pre is total protein concentration at baseline or inlet, and P post is the protein concentration at the time of blood sampling during HD or at outlet.

Statistical analysis

Statistical analysis was performed with SPSS for windows version 11.0 (SPSS, Chicago, IL, USA). Data were expressed as mean ± SD, unless otherwise mentioned. Comparisons between groups (healthy controls, stage 3–4 CKD and HD patients) were made by Kruskal–Wallis analysis of variance with Tukey's post-test or Mann–Whitney U-test (for comparisons between stage 3–4 CKD and HD groups) where appropriate. The Fisher exact test was used to compare the categorical variables. Repeated measures in HD patients were compared by Friedman test. Spearman correlation was used for correlations between MFI of TLR-2 and TLR-4 and IL-6. Stepwise multiple regression analysis was performed to define the predictors of inflammation. The following variables were included into the analysis: age, duration of HD in HD patients, serum albumin, Ca × P product, serum creatinine in stage 3–4 CKD patients or Kt/V in HD patients, and MFI of TLR-2 and TLR-4. A two-tailed P-value <0.05 was considered statistically significant.

Results

The demographic characteristics of the study populations are presented in Table 1. The mean age of the study populations and gender were similar in control, stage 3–4 CKD and HD patients. Underlying causes of CKD were similar in both stage 3–4 CKD and HD patients. Haemoglobin and serum albumin levels were lower, and phosphorus, PTH and ferritin levels were higher in HD patients as compared with stage 3–4 CKD patients.

Table 2. TLR expression patterns on monocytes and IL-6 concentrations in study populations

	Controls (n = 34)	Stage 3–4 CKD (n = 34)	HD baseline (n = 32)	HD 120 min (n = 32)	HD 300 min (n = 32)	HD 24 h (n = 32)
$CD14^+/TLR2^+$ (%)	93.6 ± 5.3	93.6 ± 8.3	90.3 ± 11.5	94.6 ± 6.4	91.2 ± 12.9	92.3 ± 10.9
TLR-2 (MFI)	1424 ± 246	1811 ± 669[*]	1837 ± 672[*]	1650 ± 578[†]	1817 ± 652	1914 ± 596[‡]
$CD14^+/TLR4^+$ (%)	93.2 ± 5.7	84.0 ± 11.8[*]	85.0 ± 12.0[*]	83.5 ± 14.3	82.4 ± 14.1	89.9 ± 12.8[†,‡,#]
TLR-4 (MFI)	817 ± 166	922 ± 329	918 ± 294	912 ± 333	892 ± 277	1117 ± 431[†,‡,#]
IL-6 (pg/mL)		9.5 ± 5.0	9.3 ± 4.5		10.6 ± 5.6[†]	9.1 ±4.9[#]

Values are expressed as mean ± SD unless indicated differently. TLR, Toll-like receptor; MFI, mean fluorescence intensity; IL-6, interleukin 6.
[*]P < 0.05 vs control group.
[†]P < 0.05 vs baseline HD group.
[‡]P < 0.05 vs HD 120 min.
[#]P < 0.01 vs HD 300 min.

Table 3. The absolute number of leucocyte subpopulations and expression of TLRs on $CD14^+$ monocytes during HD treatment in the preliminary study

	0 min	15 min	30 min	60 min	120 min	240 min	300 min	24 h
Leucocytes	6772 ± 1207	5617 ± 1076*	6321 ± 1109	7353 ± 1220†	7342 ± 1012†	7517 ± 911†,‡	7680 ± 1201†,‡	8279 ± 1198$^{*,\dagger,\#}$
Neutrophils	4541 ± 943	3650 ± 961*	4277 ± 871	5158 ± 998$^{\dagger,\#}$	5313 ± 895$^{\dagger,\#}$	5216 ± 996†,‡	5208 ± 986†,‡	4897 ± 728†
Lymphocytes	1561 ± 397¶	1387 ± 388¶	1418 ± 376¶	1444 ± 359¶	1327 ± 339¶	1493 ± 519¶	1456 ± 399 ¶	2278 ± 697¶
Monocytes	642 ± 163†	342 ± 119	408 ± 173†	501 ± 238†	480 ± 182†	607 ± 219†	728 ± 234†	787 ± 183†
$CD14^+/TLR2^+$ (%)	77.4 ± 16.6	83.7 ± 9.3	86.3 ± 10.5	84.1 ± 16.9	85.7 ± 12.6	80.8 ± 18.9	78.8 ± 17.6	79.1 ± 17.7
$CD14^+/TLR4^+$ (%)	68.0 ± 13.7	77.4 ± 15.5	73.5 ± 16.7	75.6 ± 23.4	71.8 ± 15.2	64.6 ± 21.2	67.2 ± 20.7$^{\#,\&}$	69.9 ± 14.3
TLR-2 (MFI) on $CD14^+$ monocytes	1608 ± 326	1507 ± 175	1453 ± 208	1496 ± 157	1399 ± 217	1545 ± 230	1580 ± 271	1611 ± 278$^{\dagger,\ddagger,\&}$
TLR-4 (MFI) on $CD14^+$ monocytes	782 ± 124	756 ± 96	740 ± 126	785 ± 136	817 ± 79	768 ± 81	763 ± 126	841 ± 180

Values are expressed as mean ± SD unless indicated differently. TLR, Toll-like receptor; MFI, mean fluorescence intensity.
$^{*}P < 0.05$ vs baseline.
$^{\dagger}P < 0.001$ vs 15 min.
$^{\ddagger}P < 0.05$ vs 30 min.
$^{\#}P < 0.01$ vs 30 min.
$^{\P}P < 0.001$ vs 24 h
$^{\&}P < 0.05$ vs 60 min.

Table 4. The expression TLRs on $CD14^+CD16^+$ monocytes during HD ($n = 8$)

	0 min	15 min	30 min	60 min	120 min	240 min	300 min	24 h
$CD14^+/CD16^+$ monocytes (%)	14.4 ± 5.6	5.7 ± 4.6*	6.0 ± 4.9*	8.8 ± 4.9†	12.1 ± 5.1‡	10.7 ± 2.3†	11.4 ± 1.7	12.8 ± 7.0‡
$CD14^+/CD16+/TLR2^+$ monocytes (%)	14.0 ± 4.8	3.5 ± 1.8*	5.7 ± 4.1*	8.2 ± 3.3†	11.0 ± 5.0†	9.3 ± 1.7	9.2 ± 0.5	12.1 ± 5.9$^{\#}$
$CD14^+/CD16^+/TLR4^+$ monocytes%	13.9 ± 4.4	6.0 ± 6.0*	5.1 ± 2.9*	8.4 ± 3.5†	11.4 ± 3.0¶	8.8 ± 2.9	10.5 ± 4.1	12.2 ± 5.1‡,¶
TLR-2 (MFI) on $CD14^+CD16^+$ monocytes	1841 ± 525	2098 ± 651	1776 ± 675	1722 ± 401	1604 ± 417†	1898 ± 427	1885 ± 492	1866 ± 417
TLR-4 (MFI) on $CD14^+CD16^+$ monocytes	1293 ± 226	1168 ± 331	1298 ± 481	1275 ± 547	1318 ± 403	1332 ± 460	1297 ± 443	1425 ± 424

Values are expressed as mean ± SD unless indicated differently. TLR, Toll-like receptor; MFI, mean fluorescence intensity.
$^{*}P < 0.01$ vs baseline.
$^{\dagger}P < 0.05$ vs baseline.
$^{\ddagger}P < 0.001$ vs 15 min.
$^{\#}P < 0.05$ vs 30 min.
$^{\P}P < 0.01$ vs 30 min.

The expression of TLRs on monocytes in the study populations

The percentage of $CD14^+$ monocytes expressing TLR-2 were similar in controls, stage 3–4 CKD and HD patients at baseline (Table 2). However, the percentage of $CD14^+$ monocytes expressing TLR-4 were significantly lower in stage 3–4 CKD and HD patients at baseline than in controls (Table 2).

The mean fluorescence intensities (MFI) of TLR-2 on $CD14^+$ monocytes were significantly higher in stage 3–4 CKD and HD patients at baseline than in controls (Table 2). However, the MFI of TLR-2 on $CD14^+$ monocytes were similar in HD patients at baseline and stage 3–4 CKD patients. The MFI of TLR-4 on $CD14^+$ monocytes were similar in all groups.

The expression of TLRs on $CD14^+$ monocytes in HD patients during and after HD

The results of the preliminary study in HD patients are presented in Table 3. Monocytes were depleted during the beginning phase of HD. The percentage of $CD14^+$ monocytes expressing TLR-2 and TLR-4 did not change during HD. While MFI of TLR-2 decreased significantly at 120 min of HD and it recovered back to pre-dialysis values at 24 h of HD, the MFI of TLR-4 did not change. Since we did not detect a decline in the percentage of monocytes expressing TLR-2 and TLR-4 and MFI of TLRs on these cells parallel to the monocytopaenia observed during the first 15–60 min of HD, we collected blood samples from HD patients at the beginning (baseline) and at 120 min into the HD session from their arterial lines for the remaining part of the study.

The percentage of $CD14^+$ monocytes expressing TLR-2 did not change during and after HD (Table 2). However, the percentage of TLR-4 expressing $CD14^+$ monocytes was increased at 24 h after HD compared with baseline, 120 and 300 min of HD (Table 2).

MFI of TLR-2 decreased at 120 min of HD compared with baseline (1837 ± 672 vs 1650 ± 578, $P < 0.05$), and recovered back to baseline values at 300 min and at 24 h (Table 2). MFI of TLR-4 increased at 24 h compared with baseline (941 ± 294 vs 1087 ± 441, $P < 0.05$).

Table 5. Longitudinal changes in sTLR-2 concentrations during HD at arterial inlet and venous outlet

Time	Inlet (pg/mL)	Outlet (pg/mL)
Baseline	25.6 ± 40.3	31.8 ± 52.5*
15 min	30.7 ± 47.9	43.5 ± 55.3*
30 min	35.1 ± 53.6	47.3 ± 56.2*
60 min	40.3 ± 54.9	64.4 ± 64.4*
120 min	42.7 ± 57.7	54.6 ± 67.9*
240 min	47.3 ± 63.9	57.2 ± 66.7*

*$P < 0.05$ vs inlet.

The expression of TLRs on $CD14^+CD16^+$ monocytes during and after HD by flow cytometry

To delineate whether a highly active subpopulation of monocytes, namely $CD14^+CD16^+$ monocytes, are affected by the HD procedure, and whether TLR-2 and TLR-4 expression on these cells contribute to a decline in MFI of TLR-2 on $CD14^+$ monocytes at 120 min of HD, in the second part of the study, we further analysed the expression of TLRs on $CD14^+CD16^+$ monocytes extracted from both inlet and outlet in eight HD patients (Table 4). The percentage of $CD14^+CD16^+$ monocytes expressing TLR-2 and TLR-4 were depleted during the first 60 min of HD, and they regained their baseline values at ~120 min of HD (Table 4) with a significant decline in MFI of TLR-2 ($P < 0.05$) (Table 4). In addition, the percentage of $CD14^+CD16^+$ monocytes expressing TLR-2 were similar at the inlet and outlet of the dialysers during HD, indicating that $CD14^+$ and $CD14^+CD16^+$ monocytes are not entrapped in the dialyser (data not shown).

Soluble TLR-2 levels on plasma samples

To determine whether the TLRs are shedded into circulation during HD, we measured the levels of sTLR-2 by ELISA (R&D systems, catalogue no: DY2616) in plasma samples withdrawn from eight HD patients' ($n = 8$) arterial and venous lines simultaneously. When we compared the sTLR-2 levels in arterial and venous lines, sTLR-2 values increased at venous side during HD (Table 5). These results suggest that the HD procedure might lead to shedding of TLR-2 into circulation.

Relation of TLRs expression on $CD14^+$ monocytes with serum IL-6 levels

Serum IL-6 levels as a parameter of inflammation in stage 3–4 CKD were similar to baseline IL-6 levels of HD patients (Table 2). Serum IL-6 levels significantly increased

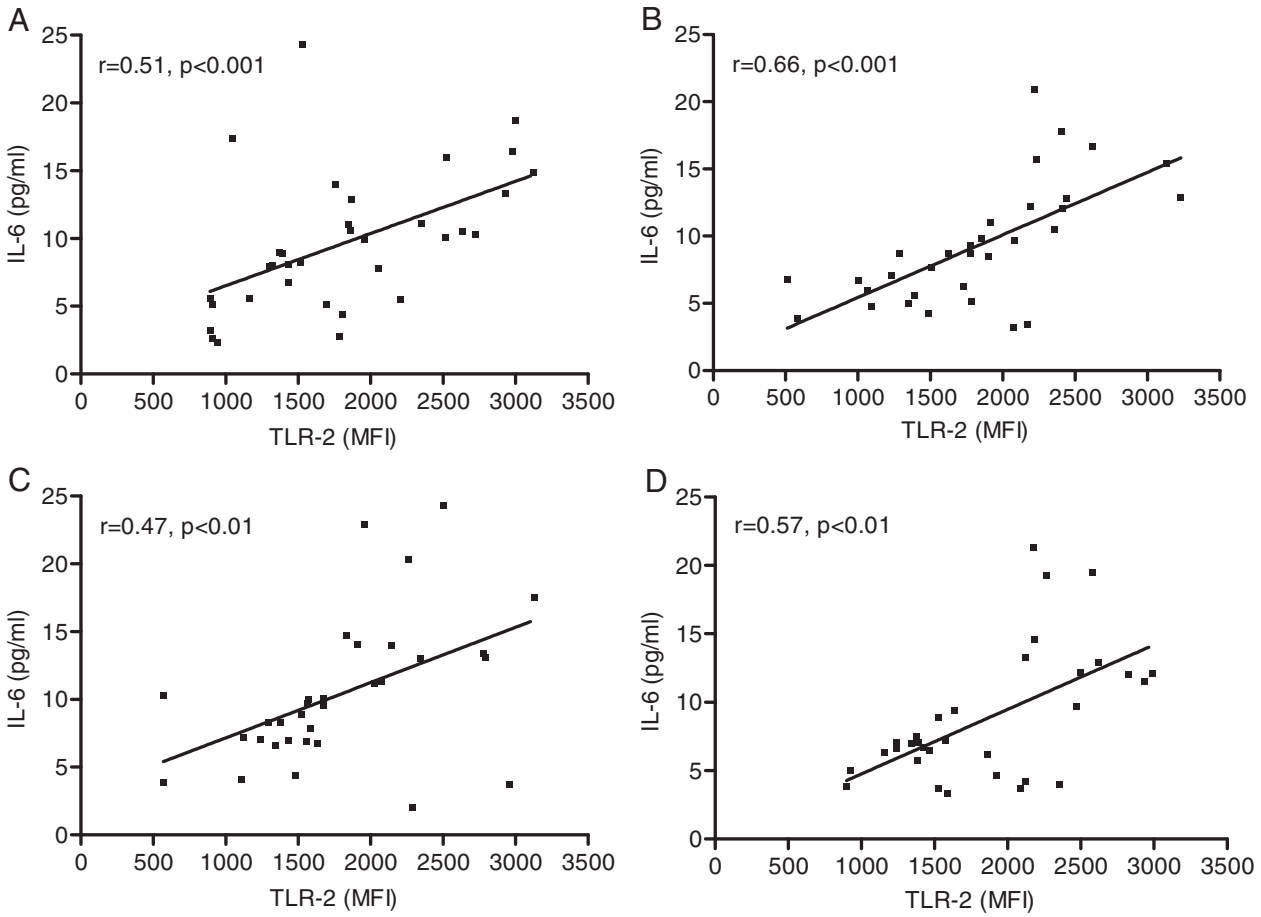

Fig. 1. Correlation between serum IL-6 and MFI of TLR-2 on $CD14^+$ monocytes in CKD and HD patients: (**A**) in stage 3–4 CKD patients, (**B**) at baseline of HD, (**C**) at 300 min of HD and (**D**) at 24 h of HD.

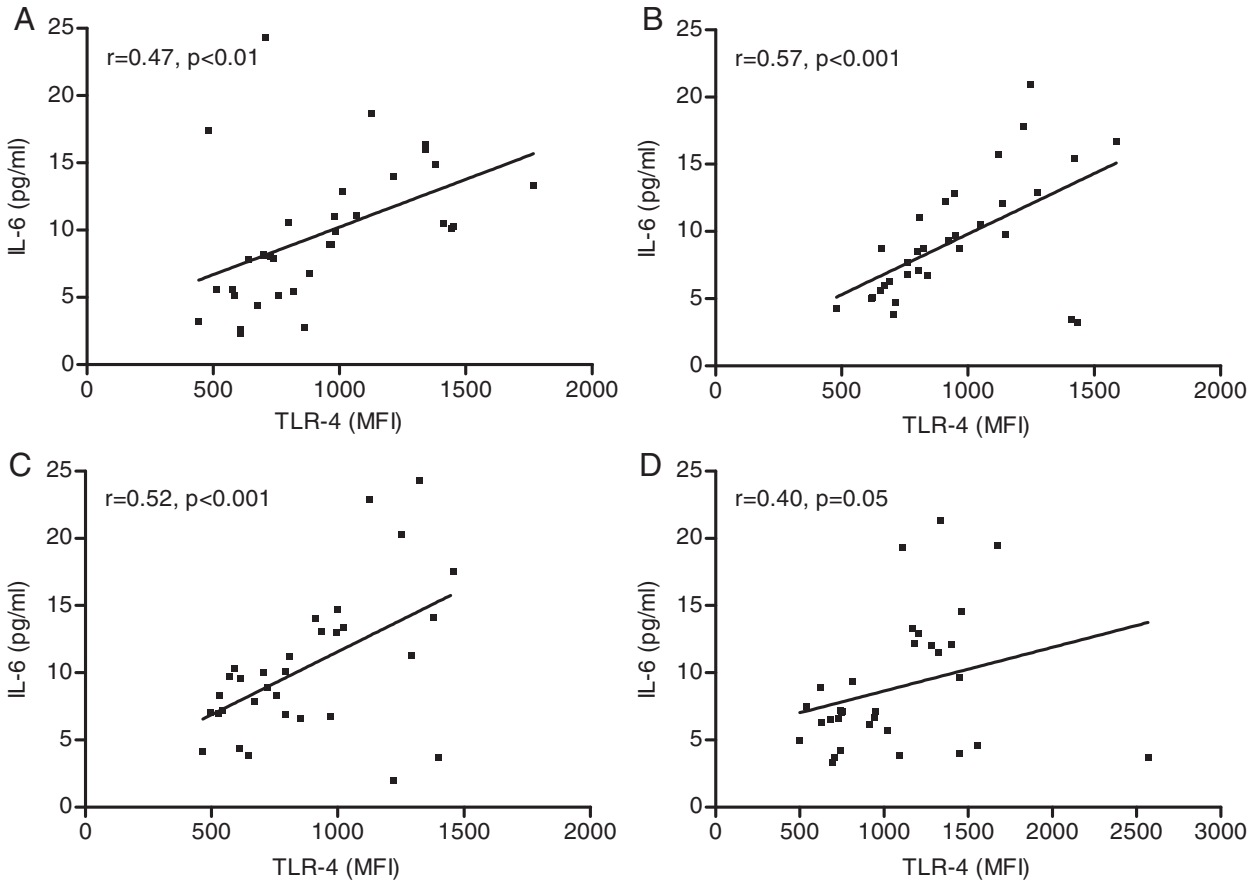

Fig. 2. Correlation between serum IL-6 and MFI of TLR-4 on $CD14^+$ monocytes in CKD and HD patients: (**A**) in stage 3–4 CKD patients, (**B**) at baseline of HD, (**C**) at 300 min of HD and (**D**) at 24 h of HD.

Table 6. Stepwise multiple regression analysis for the predictors of IL-6 concentrations in stage 3–4 CKD and HD patients at baseline, during and after HD

Patient group and model number	Dependent variable	Independent variables	β (coefficient)	*t*-test value	P-value
CKD Model-1	IL-6	MFI TLR-2	0.005	4.228	0.001
		Albumin	−4.523	−3.743	0.001
HD Model-2	IL-6 at baseline	MFI TLR-2	0.005	5.303	0.001
HD Model-3	IL-6 at 300 min	MFI TLR-2	0.005	3.656	0.002
HD Model-4	IL-6 at 24 h	MFI TLR-2	0.005	4.038	0.001

MFI, mean fluorescence intensity on $CD14^+$ monocytes; CKD, chronic kidney disease; HD, haemodialysis.

at 300 min compared with baseline (9.3 ± 4.5 vs 10.6 ± 5.6 pg/mL, $P < 0.05$) (Table 2).

Serum IL-6 levels correlated with MFI of TLR-2 and TLR-4 in stage 3–4 CKD and in HD patients at baseline, during, and after HD (Figures 1 and 2).

We modelled a stepwise multiple regression analysis to define the independent determinants of markers of inflammation in stage 3–4 CKD and HD patients (Table 6). MFI of TLR-2 and albumin were independent determinants of serum IL-6 in stage 3–4 CKD patients (model-1, $R^2 = 0.47$, $P = 0.001$). In HD patients, MFI of TLR-2 were also independent determinants of serum IL-6 at baseline (model-2, $R^2 = 0.54$, $P = 0.001$), at 300 min (model-3, $R^2 = 0.35$, $P = 0.002$) and at 24 h of HD (model-4, $R^2 = 0.40$, $P = 0.001$). However, TLR-4 was not a determinant of IL-6.

Discussion

In this study, we investigated the expression of TLR-2 and TLR-4 in stage 3–4 CKD and HD patients as compared with controls and conveyed their relation with inflammatory response. We showed that the percentages of monocytes expressing TLR-4 were lower in both CKD groups as compared with controls. MFI of TLR-2 on $CD14^+$ mono-

cytes were significantly higher in CKD patients compared with controls. While MFI of TLR-2 decreased significantly during HD, MFI of TLR-4 did not change during dialysis therapy and increased significantly 24 h after HD. In addition, the results presented here demonstrate that expression of TLR-2 on $CD14^+$ monocytes is associated with systemic inflammation in stage 3–4 CKD and in HD patients before and after completion of a HD session.

To the best of our knowledge, there are only two publications for the time being evaluating the expression of TLR-2 or TLR-4 in pre-dialysis CKD patients. In the study by Ando *et al*., a decrease of TLR-4 was found on unstimulated monocytes in CKD patients compared with healthy controls [7]. Similarly, we also found a lower percentage of TLR-4 expressing $CD14^+$ monocytes in stage 3–4 CKD and HD patients. The lower percentage of $CD14^+$ monocytes expressing TLR-4 could be compatible with uraemia per se as reported by Ando *et al.* [7]. However, these authors did not include dialysis patients. In a similar study by Kuroki *et al*., using unstimulated monocytes, no differences were found in MFI of TLR-2 between HD patients and healthy controls, whereas MFI of TLR-4 were lower in HD patients [8]. However, the percentage of monocytes expressing TLR-2 or TLR-4 were not reported. In our study, we found that MFI of TLR-2 were higher on $CD14^+$ monocytes in stage 3–4 CKD and HD patients compared with controls. It is possible to speculate that higher MFI values of TLR-2 may be related to chronic low-grade monocyte activaton in these patients [7].

In our study, >85% of monocytes in both CKD groups expressed TLR-2 and TLR-4. Even though Ando *et al*. reported that only ~5.5%, 1.5% and 3.6% of monocytes expressed TLR-4 in healthy controls, uraemic patients with prior infections and uraemic patients without prior infections, respectively, other studies have reported ~60% TLR-4 expression on unstimulated monocytes in healthy controls [13]. In the present study, we found a 93.6% TLR-4 expression on $CD14^+$ monocytes in healthy controls. It should be noted that Ando *et al*. used a different brand of TLR antibodies compared with the present study [7].

None of the above studies evaluated TLR-2 and TLR-4 expression during and after dialysis. We found no increase in the expression of TLR-2 and TLR-4 during dialysis, and for MFI of TLR-2, there was even a decrease at 120 min of HD (Table 2). One would expect downregulation of MFI of TLR-2 on $CD14^+$ monocytes as a result of entrapment of activated monocytes in the pulmonary circulation or loss into the extracorporeal circulation during HD [13–17]. In our preliminary study, we could not demonstrate a decrease in the percentage of $CD14^+$ monocytes expressing TLR-2 or TLR-4 at the beginning of the HD session (Table 3). The expression of TLR-4 on $CD14^+$ monocytes was lower in the preliminary study compared with the TLR-4 expression on $CD14^+$ monocytes in the second part of the study (Tables 2 and 3). However, the HD populations in the preliminary and in the main study were not the same. This difference may be attributed to the small sample size of the preliminary study. Depletion of another subpopulation of monocytes, namely $CD14^+CD16^+$ monocytes, could result in a decline in MFI of TLR-2. These monocytes are highly active inflammatory monocytes, are increased in HD patients compared with CKD patients and are depleted during HD [14]. To delineate whether $CD14^+CD16^+$ monocytes expressing TLR-2 are affected by the HD procedure, we further analysed the expression of TLRs on $CD14^+CD16^+$ monocytes extracted from both the inlet and outlet in eight HD patients. (Table 4). The percentage of $CD14^+CD16^+$ monocytes expressing TLR-2 were depleted during the first 60 min of HD, and they regained their baseline values at ~120 min of HD with a decline in MFI of TLR-2 (Table 4). In addition, the percentage of both $CD14^+$ and $CD14^+CD16^+$ monocytes were similar at arterial inlet and venous outlet of the dialyser (data not shown). These results might indicate that entrapment of monocytes in the dialyser may not participate in the decline of MFI of TLR-2.

Initial downregulation of TLR-2 in our study during HD could also be secondary to the shedding of TLRs into the circulation. In a subpopulation of eight HD patients, we found that sTLR-2 levels increased in outlet behind the filter compared with inlet at different time points of HD (Table 5). These results suggest that the HD procedure might lead to the shedding of TLR-2 into circulation leading to a decrease in MFI of TLR-2 on monocytes during HD.

On the other hand, we found a late (24 h) upregulation of TLR-4. The pattern of TLR-2 and TLR-4 expression during and after HD also did not evolve in parallel. While TLR-2 was downregulated at mid-dialysis, TLR-4 was not, and while TLR-4 was upregulated post-dialysis at 24 h, TLR-2 was not. Explanation for different time patterns for TLR-2 and TLR-4 is not clear based on our results. The behaviour of TLR-2 and TLR-4 expression may not be similar to each other in pathological conditions. For example, Kuroki *et al*. reported lower MFI of TLR-4 but similar TLR-2 values in HD patients compared with controls [8]. We speculate in haemodialysis patients that the ligands of TLRs such as heat shock proteins may be elevated during and after HD and may activate their receptors at different time points during and after HD [15,16]. Additionally, late upregulation of TLR-4 may also be due to activation of monocytes during HD or induction by inflammatory cytokines secreted during HD [17–20].

One of the novel aspects of this study is that we evaluated the relationship between TLR and inflammatory parameters at different stages of CKD and at different time points during and after HD. At stepwise multivariate analysis, MFI of TLR-2 were independent determinants of IL-6 concentrations before and after a HD session (Table 6). To the best of our knowledge, no other study evaluated this question in a haemodialysis population. In the study by Ando *et al*., unstimulated monocytes were not submitted to correlation analysis [7]. However, in a study in patients with rheumatoid arthritis, Iwahashi *et al*. found results in parallel with ours by demonstrating correlation between TLR-2 expression in unstimulated monocytes and CRP levels [21]. Also, several other studies have shown a similar correlation between TLR-4 expression on unstimulated monocytes and CRP levels in patients with acute infections and $HLA\text{-}B27^+$ ankylosing spondylitis, respectively [22,23]. The correlation between inflammatory parameters and TLR expression in CKD might be related to several factors, such as activation of monocytes through mechanical contact with

membranes, uraemic toxin retention, chronic stimulation by angiotensin II or increased intestinal permeability to LPS as a result of fluid overload [18,19,24–26]. Another possible mechanism could be dialysate contamination, either by LPS, peptidoglycan or DNA fragments [27,28]. However, performance of the LAL test on the dialysate at the day of the examination revealed LPS levels <0.25 mIU/mL, which is insufficient to cause monocyte activation (data not shown). In addition, a novel biological test method to assess the potential to induce IL-1β production of THP-1 cells, which is also sensitive to non-LPS pro-inflammatory elements such as peptidoglycans and deoxynucleotides, also revealed no activity in our dialysate samples (data not shown) [10]. Therefore, it is conceivable that other mechanisms, such as leucocyte activation due to shear stress or complement activation, might be at play [3,19,29]. Even so-called biocompatible membranes have the capacity to activate leucocytes [30,31].

There are some limitations to this study. Firstly, the sample size was relatively small. Secondly, we did not evaluate the expression of these molecules at mRNA levels. Thirdly, we evaluated unstimulated leucocytes, while many other studies evaluate TLR expression after stimulation with substantial amounts of LPS and/or peptidoglycan. However, we preferred to use non-stimulated monocytes because we believe they are more representative for the *in vivo* condition. One might argue that stimulation with LPS brings monocytes to a pro-inflammatory condition as seen in uraemia [24,32]. However, LPS concentrations in the experiments mentioned above were almost always very high, rather mimicking acute septic conditions than the mitigated chronic inflammatory state of uraemia [33,34].

In conclusion, this cross-sectional study showed that the expression patterns of TLR-2 and TLR-4 in stage 3–4 CKD and HD patients are associated with inflammation as assessed by serum levels of IL-6. TLR-2 expression on monocytes decreases during HD which may be secondary to shedding of this molecule during HD. Further studies are needed to investigate whether the role of TLR-2 expression on inflammatory response is specific for CKD patients and to elucidate factors triggering TLR-2 expression in CKD patients.

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Conflict of interest statement. None declared.

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Tachycardia as a predictor of poor survival in chronic haemodialysis patients

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Abstract

Background. High pulse rate is a culprit of all causes of death in the general population, but its relation to death in haemodialysis (HD) patients has not been examined in a large patient cohort.

Methods. We examined the relationship between pulse rate (beats per minute, bpm) before an HD session and survival based on the nationwide HD registry of the Japanese Society for Dialysis Therapy. Outcomes were confirmed using the coded ID numbers of both 2005 and 2006 registries. Logistic analyses were performed to determine the effect of pre-HD pulse rate on survival. A total of 147 702 patients (50.5% men; 31.4% with diabetes mellitus; mean age 63.6 years) on HD three times weekly were studied. Mean (SD) pulse rate was 74.6 (12.0) bpm.

Results. The pulse rate distribution was as follows: 0.7% (40–49 bpm), 6.1% (50–59 bpm), 25.3% (60–69 bpm), 38.1% (70–79 bpm), 18.7% (80–89 bpm), 7.9% (90–99 bpm), 2.4% (100–109 bpm) and 0.7% (110–129 bpm). Overall 1-year mortality rate was 6.6%. Compared with the reference pulse rate (60–69 bpm), the odds ratio (95% CI) for 1-year mortality was 1.20 (0.88–1.63, NS: 40–49 bpm), 1.06 (0.93–1.21, NS: 50–59 bpm), 1.13 (1.04–1.22, P = 0.0037: 70–79 bpm), 1.46 (1.33–1.60, P < 0.0001: 80–89 bpm), 1.91 (1.70–2.15, P < 0.0001: 90–99 bpm), 2.61 (2.19–3.10, P < 0.0001: 100–109 bpm), and 2.43 (1.79–3.30, P < 0.0001: 110–129 bpm) after adjusting for age, sex, diabetes mellitus, body mass index, HD duration, serum albumin, haemoglobin, systolic blood pressure, medication for hypertension, and history of acute myocardial infarction.

Conclusions. Survival rate decreased with an increase in the pre-HD pulse rate in chronic HD patients. The causality of this association and the reasons for a better annual mortality rate of 6.6% remain to be clarified.

Keywords: cardiovascular disease; chronic haemodialysis; pulse rate; survival

Introduction

Pulse rate is usually measured with blood pressure in patients undergoing chronic haemodialysis (HD). Both pulse