View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

http://www.kidney-international.org
© 2006 International Society of Nephrology

Reduced expression of Toll-like receptor 4 contributes to impaired cytokine response of monocytes in uremic patients

M Ando[1], A Shibuya[1], K Tsuchiya[2], T Akiba[2] and K Nitta[2]

[1]Department of Nephrology, Tokyo Metropolitan Komagome Hospital, Bunkyo-Ku, Tokyo, Japan and [2]Department of Medicine, Kidney Center, Tokyo Women's Medical University, Shinjuku-Ku, Tokyo, Japan

Toll-like receptors (TLRs) play a pivotal role in pathogen recognition and subsequent cytokine synthesis by immune cells. Uremic patients have a high infectious morbidity, but it remains unclear if this arises from the defective innate immune responses related to TLRs. We studied TLR4 expression in monocytes and their intracellular cytokine synthesis in response to lipopolysaccharide (LPS) stimulation in 35 predialysis patients with chronic kidney disease (CKD) with or without predisposition to bacterial infections and 16 age-matched controls. Expression of TLR4 in unstimulated peripheral monocytes was determined by staining with anti-TLR4 antibody and analysis with flow cytometry. Monocytes were then stimulated by LPS, labeled with anti-CD14 antibody, and subjected to intracellular cytokine staining and flow cytometry. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 synthesis was examined in CD14$^+$ monocytes. TLR4 expression was constitutively diminished in CKD patients with reduced expression being more severe in those CKD patients who were predisposed to infections. Monocytes from these infection prone CKD patients exhibited significantly reduced synthesis of TNF-α, IL-1β, IL-6, and IL-8 in response to LPS challenge compared with those from control subjects. The intensity of synthesis of each cytokine significantly correlated with TLR4 expression levels in monocytes ($P<0.01$). The capacity of monocytes to synthesize proinflammatory cytokines was significantly reduced in infection prone CKD patients, and this may possibly be due to the reduced monocyte expression of TLR4. Abnormal TLR4 expression by monocytes may play a role in the susceptibility of such patients to bacterial infections.

Kidney International (2006) **70,** 358–362. doi:10.1038/sj.ki.5001548; published online 31 May 2006

KEYWORDS: innate immunity; proinflammatory cytokines; intracellular cytokine staining; lipopolysaccharide; flow cytometry

Correspondence: *M Ando, Department of Nephrology, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-Ku, Tokyo 113-8677, Japan. E-mail: nephrol@cick.jp*

Received 28 October 2005; revised 16 March 2006; accepted 22 March 2006; published online 31 May 2006

Patients with chronic kidney disease (CKD) as well as dialysis patients have an increased incidence of infections,[1] and this suggests that innate defense systems are impaired in uremic patients. Microbial recognition and subsequent release of proinflammatory cytokines are critical features of the innate host defense system against microorganisms. Members of the Toll-like receptor (TLR) family are responsible for the recognition of pathogen-associated molecular patterns expressed by a wide spectrum of infectious agents. TLRs activate the nuclear factor-κB pathway through several adaptor molecules, and therefore regulate cytokine expression. Activation of the nuclear factor-κB pathway links the innate and adaptive immune response via the production of inflammatory cytokines and the induction of costimulatory molecules.[2] Although at least 10 TLRs have been identified in humans, TLR4 is a key player as it recognizes lipopolysaccharide (LPS) of Gram-negative bacteria bound to CD14. Ligation of TLR4 induces production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8. Consequently, the expression levels of TLR4 may be involved in regulating the propensity of immune cells to cytokine synthesis following LPS stimulation and initiating a response that effectively eliminates Gram-negative bacterial infections.[3,4] However, the level of TLR4 expression and its involvement in cytokine response have not been studied in CKD patients.

It is becoming increasingly clear that the appropriate induction of cytokines by immune cells is necessary for the full development of the innate host defense. Cytokines exert their major physiologic and pathophysiologic effects as autocrine or paracrine factors. Therefore, altered local processing and release of cytokines are very relevant in the pathogenesis of innate immune dysfunction. The study of circulating cytokine levels has limited value and gives little information as a result of their short half-lives and important local effects.[5] In contrast, measuring the capacity of peripheral blood cells to synthesize cytokines in response to pathogenic stimuli may provide a more precise and relevant picture in clinical practice.[5–7]

To elucidate the effects of uremia upon the capacity of innate host defense toward an infectious challenge, we

investigated constitutive expression of TLR4 in peripheral monocytes and the intracellular synthesis of proinflammatory cytokines in response to LPS stimulation. It is well described that dialysis may significantly influence cytokine production whereas the predisposition toward infections varies among individuals even within the uremic population. Thus, in this study, we focused on predialysis CKD patients who had been susceptible to bacterial infectious diseases.

RESULTS

Laboratory data of the subjects

The laboratory data of the subjects are shown in Table 1. There were no significant differences in all laboratory variables between the CKD patients with prior bacterial infections and those without antecedent infections. The CKD and control groups were comparable with respect to total white blood cell, monocyte, and lymphocyte counts. The hematocrit was significantly lower in the CKD patients. The serum TNF-α, IL-1β, IL-6, and IL-8 levels were not different between the CKD and control groups. As expected, significant differences were observed between the CKD and control groups with regard to serum parameters that are related to uremia (estimated glomerular filtration rate, urea nitrogen, creatinine, and β_2 microglobulin levels), but there were no differences apparent with regard to nutritional parameters (total protein, albumin, and lipids levels). The erythrocyte sedimentation rate was significantly higher possibly due to anemia in the CKD patients but C-reactive protein levels were not different.

Table 1 | Laboratory data

	CKD (*n*=35)	Control (*n*=16)	*P*-values
WBC counts (/μl)	5038±1565	5258±1247	NS
Monocytes (/μl)	427±122	435±88.0	NS
Lymphocytes (/μl)	1348±665	1532±888	NS
Hct (%)	30.9±7.1	44.5±4.4	$P<0.05$
Serum data			
TP (g/dl)	6.8±1.4	7.0±1.3	NS
Alb (g/dl)	3.9±1.2	4.3±0.7	NS
UN (mg/dl)	68.1±9.3	11.8±3.2	$P<0.01$
Cr (mg/dl)	5.9±4.1	0.84±0.3	$P<0.01$
β_2MG (mg/dl)	12.7±3.56	0.91±0.14	$P<0.01$
TC (mg/dl)	196±23.2	236±69.9	NS
TG (mg/dl)	131±113.6	104.5±20.2	NS
ESR (mm/h)	17.1±8.78	8.50±2.22	$P<0.05$
CRP (mg/dl)	0.2±0.1	0.1±0.2	NS
Estimated GFR (ml/min/1.73 m^2)	15.4±2.67	84.8±4.65	$P<0.01$
Serum cytokine levels			
TNF-α (pg/ml)	25.1±20.2	23.0±12.7	NS
IL-1β (pg/ml)	2.11±2.74	1.98±3.03	NS
IL-6 (pg/ml)	4.31±2.00	3.87±2.21	NS
IL-8 (pg/ml)	308±275	297±286	NS

Alb, albumin; β_2MG, β_2 microglobulin; CKD, chronic kidney disease; Cr, cratinine; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; GFR, glomerular filtration rate; Hct, hematocrit; IL, interleukin; NS, nonsignificant; TC, total cholesterol; TG, triglycerides; TNF, tumor necrosis factor; TP, total protein; UN, urea nitrogen; WBC, white blood cell.
GFR is estimated by the Modification of Diet in Renal Disease equation.
Data are expressed as mean±s.d.

Baseline expression of TLR4 in unstimulated peripheral monocytes

We investigated the constitutive expression of TLR4 in monocytes from 35 CKD patients and healthy controls. TLR4 expression was downregulated in CKD patients compared with controls. The frequency of TLR4 positive cells (% of positive cells) and the level of expression (mean fluorescence intensity (MFI)) were significantly higher in control subjects (frequency: 5.52±0.518% and MFI: 3.83±1.68) than in CKD patients. This difference was more obvious in the 20 CKD patients with prior recurrent bacteria infections (frequency: 1.35±1.68% and MFI: 1.03±0.0603) than in the 15 CKD patients without prior infections (frequency: 3.61±1.41% and MFI: 2.62±1.00). The data are shown in Figure 1.

Intracellular cytokine induction by monocytes in response to LPS

This examination was performed in CKD patients ($n=20$) who had been predisposed to bacterial infections and age-matched healthy controls ($n=16$). There were no differences in the intracellular monocyte cytokine levels between the two groups in the absence of LPS stimulation as previously described.[8] However, significant differences were detected between groups following LPS stimulation. Table 2 shows the percentage of cells that were positive for TNF-α, IL-1β, IL-6, and IL-8 (frequency), and the level of intracellular cytokine synthesis expressed by MFI (intensity) in the CKD patients and controls. The CKD patients exhibited a significantly reduced frequency and intensity in the synthesis of all the cytokines tested compared with the controls.

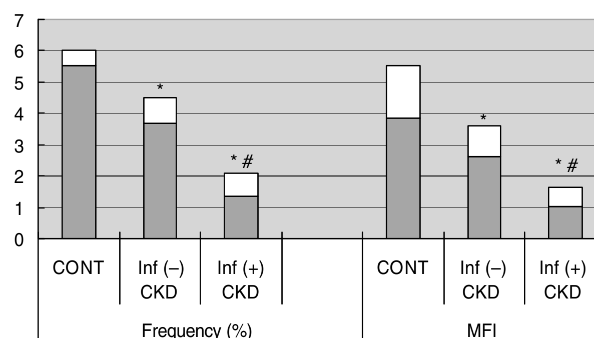

Figure 1 | Surface expression of TLR4 in unstimulated monocytes in CKD patients and age-matched control subjects (CONT). The percentage of cells that are positive for anti-human TLR4 specific mAb (frequency) is shown on the left side. Their MFI is on the right side. The data are shown as mean (lower black column)±s.e. (upper white column). *P*-values represent the comparison between CONT ($n=16$) and CKD ($n=35$). Inf (+) CKD means CKD with prior recurrent infections ($n=20$). Inf (–) CKD means CKD without prior recurrent infections ($n=15$). *$P<0.01$ compared with CONT. $^{\#}P<0.01$ compared with Inf (–) CKD.

Correlation of intracellular cytokine synthesis and TLR4 expression in monocytes

Intracellular synthesis of each cytokine was significantly correlated with intensity of TLR4 expression in CKD patients ($n = 20$) on bivariable analysis. In addition, the synthesis of all cytokines except IL-8 was significantly correlated with the frequency of TLR4 expressing cells (Table 3). The frequency of cytokine producing cells had no significant correlation with TLR4 expression. Cytokine synthesis had no significant correlation with other clinical variables associated with renal function such as serum creatinine, urea nitrogen, β_2 microglobulin, and estimated glomerular filtration rate.

DISCUSSION

This study has focused upon the impairment of innate immunity related to local cytokine responses in predialysis CKD patients and the possible role of abnormal TLR4 expression. First, a significant reduction of TLR4 expression was evident in CKD patients irrespective of predisposition to bacterial infection, but it was more severe in CKD patients with prior bacterial infections. Secondly, we found that LPS-induced cytokine synthesis was impaired in the CKD patients predisposed to bacterial infections. Finally, each cytokine response exhibited a significant correlation with the expression level of TLR4 in monocytes. These findings suggest that the significant reduction of TLR4 expression in monocytes in CKD patients who are susceptible to infections may underlie the impaired local cytokine synthesis by monocytes in response to Gram-negative microorganisms.

Table 2 | Frequency and intensity of cytokine synthesis by monocytes

Cytokines	CKD (*n*=20)	Control (*n*=16)	*P*-values
TNF-α (%)	12.1±14.7	25.6±16.7	<0.01
TNF-α (MFI)	2.62±0.602	3.47±0.904	<0.01
IL-1β (%)	10.6±20.6	29.6±26.2	<0.05
IL-1β (MFI)	1.33±0.249	1.72±0.569	<0.05
IL-6 (%)	3.12±2.97	9.50±3.64	<0.01
IL-6 (MFI)	1.75±0.363	2.31±0.893	<0.01
IL-8 (%)	18.5±21.9	30.7±33.6	<0.01
IL-8 (MFI)	1.42±0.337	1.85±0.770	<0.05

CKD, chronic kidney disease; IL, interleukin; MFI, mean fluorescence intensity; TNF, tumor necrosis factor.
P-values represent the comparisons between CKD and control.
Data are expressed as mean±s.d.

Table 3 | Correlation between intensity of cytokine synthesis and TLR4 expression in CKD patients

Cytokine	Correlation coefficient (*n*=20)	*P*-value
TNF-α	0.825 (0.743)	0.0019 (0.0106)
IL-1β	0.779 (0.700)	0.0058 (0.0216)
IL-6	0.846 (0.835)	0.0010 (0.0014)
IL-8	0.745 (0.617)	0.0110 (0.0568)

CKD, chronic kidney disease; IL, interleukin; TLR4: Toll-like receptor 4.
Data in parenthesis indicate correlation coefficient and *P*-value for frequency of TLR4 expression.

To date, the factors that decrease TLR expression *in vivo* are poorly understood. While Tamandl *et al.*[9] have shown that TNF-α reduces and IL-6 enhances TLR4 expression on monocytes, our data did not show any difference in serum levels of these cytokines between the CKD and control subjects (Table 1). Also, these serum cytokine levels did not exhibit any statistical correlation with TLR4 expression on bivariable analysis (data not shown). To clarify whether previous infections result in a residual immune deficit that affects TLR4 expression, we compared TLR4 expression between CKD patients with and without a predisposition to bacterial infections. Our data indicate that the reduction in TLR4 expression in CKD patients compared with healthy controls occurs irrespective of the presence or absence of prior bacterial infections (Figure 1). This suggests that the decreased TLR4 expression is secondary to uremia *per se* and is not the result of previous recurrent infections. However, our study did not clarify the cause of the further reduced TLR4 expression in CKD patients with a history of prior recurrent infections. Genetic variations in individual TRL4 expression might be involved in this difference.

We studied the capacity of monocytes to synthesize proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 in response to LPS stimulation in the CKD patients who were susceptible to infections. Our results showed that cytokine synthesis was significantly reduced in these CKD patients compared with the control subjects. In exerting their major physiologic and pathophysiologic effects, cytokines act as autocrine or paracrine entities. An inadequate or delayed proinflammatory cytokine release in the local environment may limit the immune response and increase the risk of clinically significant infections.[4,10,11] As a consequence of the critically important local effects of cytokines and the short-half-life of monokines, the study of blood levels is of limited value.[5] Indeed, several studies have demonstrated a correlation between reduced proinflammatory monokine synthesis in response to stimuli and a higher mortality in septic patients even though plasma monokine levels are elevated.[12,13] This study did not address anti-inflammatory cytokine profiles such as IL-10 synthesis and the ratio of IL-10 to TNF-α, which also need to be considered in host defense.[14,15] We believe, however, that the reduction of proinflammatory monokines is directly relevant to the susceptibility to bacterial infections.[10–13]

To the best of our knowledge, there is little known about the mechanisms regulating the production of cytokines by peripheral blood monocytes in response to infectious stimuli under chronically uremic conditions. We suggest that the altered cytokine response to LPS is the result of abnormal TLR4 expression since LPS binding to TLR4 triggers monocyte cytokine production.[2–4] Although the exact mechanism is currently unclear, antibodies to TLR4 inhibit LPS-induced cytokine production by human peripheral blood mononuclear cells, human gingival fibroblasts, and murine peritoneal macrophages.[16,17] In fact, the clinical relevance of TLR4 expression levels is suggested by the

significant correlation with *in vitro* cytokine response at the time of LPS stimulation in the CKD patients (Table 3).

In conclusion, CKD patients who are predisposed to bacterial infections exhibit inadequate innate host defense with respect to their ability to generate appropriate pro-inflammatory cytokines in response to invading microorganisms. This compromised cytokine response could be associated with their severely decreased TLR4 expression.

MATERIALS AND METHODS

Patients

The study included 35 predialysis CKD patients (male/female, 20/15; mean age, 62.3 ± 6.31 years) and 16 age-matched healthy subjects (male/female, 8/8; mean age, 61.3 ± 6.11 years). Twenty CKD patients had been hospitalized on at least two occasions for treatment of infectious diseases such as pneumonia ($n = 32$), urinary tract infections ($n = 4$), sepsis ($n = 4$), phlegmonous cellulitis ($n = 4$), and cholecystitis ($n = 2$). The shortest time between the last hospital discharge and entry into the study was 2 months and 11 days (the mean time was 3.8 ± 1.8 months). Fifteen CKD patients had no such medical history of infections. The mean time from diagnosis of CKD to the study entry in 35 patients was 4.6 ± 3.2 years. Eight out of 35 CKD patients had received recombinant human erythropoietin administration but not iron therapy. The dose of recombinant human erythropoietin was 200–400 U/kg per month. The chronic renal failure of the CKD patients was due to the following diseases: nephrosclerosis ($n = 23$) and chronic glomerulonephritis ($n = 12$). Any patient that had renal failure due to collagen diseases, diabetes mellitus, and hepatitis C was excluded from the study since the immune cells of such patients are considered to be functionally abnormal. None of the subjects took any drugs that can influence the immune system. The study was approved by the institutional review board of the hospital, and was conducted in accordance with the Declaration of Helsinki Principles. Informed consent was obtained from all subjects.

Reagents and antibodies

Brefeldin-A and LPS were purchased from Sigma Chemicals (Tokyo, Japan). The following reagents were purchased from Becton Dickinson (CA, USA): fluorescence-activated cell sorter Lysing Solution®, Permeabilizing Solution®, fluorescein isothiocyanate (FITC)-conjugated anticytokine monoclonal antibodies (mAbs) to TNF-α (anti-human TNF-α FITC) and IL-6 (anti-human IL-6 FITC), PE-conjugated anti-cytokine mAbs to IL-1β (anti-human IL1β PE) and IL-8 (anti-human IL-8 PE), and isotype-matched immunoglobulins (IgGs) of irrelevant specificities (FITC- or PE-labeled mouse IgG). PC5-conjugated mAb to CD14 was purchased from Immunotech (Tokyo, Japan). PE-labeled anti-human TLR4 mAb (HTA-125, mouse IgG_{2a}) was obtained from Santa Cruz Biotechnology (CA, USA). RPMI 1640 medium, phosphate-buffered saline, paraformaldehyde, and bovine serum albumin were purchased from GIBCO (Tokyo, Japan). enzyme-linked immunosorbent assay kits for measurement of serum TNF-α, IL-1β, IL-6, and IL-8 levels were purchased from R&D Systems (Quantikine™ Human Cytokine Immunoassay; Minneapolis, MN, USA).

Blood collection and measurements

Blood samples were collected from the predialysis CKD patients and control subjects before having breakfast in the morning. Blood cell count and routine laboratory data were obtained by using the automated analyzer SF-3000 Sysmex (Tokyo, Japan). The serum cytokine levels were measured by enzyme-linked immunosorbent assay kits following the manufacturer's protocol. We used the four-variable (abbreviated) modification of diet in renal disease equation to estimate the glomerular filtration rate.

Measurement of baseline expression of TLR4 on unstimulated monocytes

One hundred micro-liter of peripheral blood was incubated with anti-TLR4 PE mAb and anti-CD14 FITC mAb for 30 min at 4°C. The labeled blood was automatically lysed, washed, and resuspended in 500 μl of phosphate-buffered saline (TQ-prep™ Beckman Coulter). Samples were analyzed using a flow cytometer (Cytomics™FC500 Beckman Coulter, CA, USA) and the data were assessed with Cytomics RXP (Beckman Coulter). The light scatter and fluorescence channels were set at a logarithmic gain to identify the monocyte population by cell size and granularity (gate R1). Cells that were positive for CD14 were gated on the side-scatter versus fluorescence 1 (SSC-FL1) plot (gate R2). The analysis below was performed on 3000 CD 14^+ monocytes within both R1 and R2 gates. The cells labeled with anti-TLR4 PE mAb and anti-CD14 FITC mAb were profiled on the FL1–FL2 plot and compared to samples treated with anti-CD14 FITC mAb and isotype-matched irrelevant IgG PE (negative controls). Cutoff quadrant markers were set individually for each measurement based on the negative control. The percentage of cells that were positive for anti-TLR4 mAb (frequency) and their MFI were calculated on the histogram for the assessment. All experiments were performed in duplicate and their mean values were used for analysis. The representative figures showing the expression of TLR4 from a control subject are demonstrated in Figure 2.

Intracellular cytokine staining

To measure cytokine production following cell stimulation, we employed an intracellular cytokine staining method. We used the FASTIMMUNE Assay System® kit (Becton Dickinson) based on the method described by Jung *et al.*[6] and Picker *et al.*[6] The optimal dose of LPS and stimulation time for cytokine synthesis had been determined by a previously published *in vitro* time course study.[8,18] In brief, 1 ml of peripheral blood was diluted with 1 ml of RPMI 1640 medium and then incubated with 15 μg/ml of brefeldin-A. This disrupts the intracellular Golgi-mediated transport and allows cytokines to accumulate within the cell thereby enhancing the cytokine signal. Thereafter, 0.1 μg/ml of LPS was added to activate the monocytes and the blood was then incubated for 4 h at 37°C in a CO_2 incubator (5% CO_2). The viability of mononuclear cells (>95%) in whole blood was evaluated using Trypan blue exclusion. Thereafter, 500 μl of the activated blood was labeled with anti-CD14 PC5 mAb for 15 min at room temperature.

Fluorescence-activated cell sorter lysing solution® (4 ml) was added to the activated, labeled blood and incubated for 10 min. The sample was then washed twice with phosphate-buffered saline/0.1% bovine serum albumin by centrifugation and the supernatant was removed. Permeabilizing solution® (500 μl) was added and the specimen was left for 10 min in the dark. After washing the cells twice, cell pellets were incubated with the fluorescent anticytokine mAbs in 50 μl of RPMI medium for 30 min in the dark. After washing the cells by centrifugation, the supernatants were removed and cells were fixed in 500 μl of phosphate-buffered saline/1% paraformaldehyde before being analyzed by flow cytometry. Various intracellular cytokines including TNF-α, IL-1β, IL-6, and IL-8 were

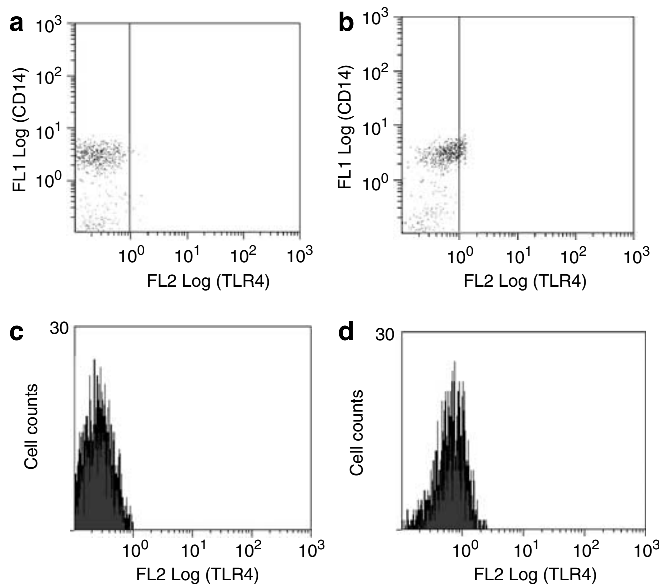

Figure 2 | Flow cytometric analysis of TLR4 in peripheral monocytes. The light scatter and fluorescence channels were set at a logarithmic gain to identify monocyte population by cell size and granularity. Cells that were positive for CD14 FITC mAb were gated on the SSC-FL1 plot as described in Materials and Methods. The cells labeled with anti-TLR4 PE mAb and anti-CD14 FITC mAb were profiled on the FL1–FL2 plot and compared to the samples that had been treated with anti-CD14 FITC mAb and isotype irrelevant IgG PE ((**a**) control IgG and (**b**) TLR4 mAb). Cutoff quadrant markers based on the negative control were set individually for each measurement. The percentage of cells that were positive for anti-TLR4 mAb (frequency) and their MFI were calculated on the histogram for the assessment ((**c**) control IgG and (**d**) anti-TLR4 mAb).

detected by specific mAbs. Isotype control IgGs of irrelevant specificities were added at matching concentrations in control samples to determine the nonspecific binding of the mAbs to the cells.

Intracellular cytokine measurement by flow cytometry

The samples were analyzed by using a flow cytometer (Cytomics™FC500) and the data were assessed with Cytomics RXP. The light scatter and fluorescence channels were set at a logarithmic gain to identify monocyte population by cell size and granularity (gate R1). Only cells that were positive for CD14 PC5 mAb were gated on the SSC-FL3 plot (gate R2). Each analysis was performed on 5000 CD 14^+ monocytes within both R1 and R2 gates. The cells labeled with specific anticytokine mAbs were profiled on the FL1–FL2 plot and compared to samples treated with isotype-matched irrelevant IgGs (negative controls). Cutoff quadrant markers based on the negative control were set individually for each measurement. The percentage of cells that were positive for each cytokine antibody (frequency) was calculated on the quadrant diagram for the assessment as described previously.[8] The MFI was also measured to determine the level of cytokine synthesis. All experiments were performed in duplicate and their mean values were used for analysis.

Statistics

The data are expressed as mean ± s.d. unless otherwise stated. Statistical analysis was performed using the Mann–Whitney *U*-test to compare data between the two study groups and using the Kruskal–Wallis test between the three study groups. $P < 0.05$ was considered to be statistically significant. Bivariable analyses exploring the relationship of cytokine synthesis to variables were performed using the nonparametric Spearman rank correlation test.

ACKNOWLEDGMENTS

We sincerely thank Dr Yoko Ando in the Clinic of the Kantoh-Shinetsu National Tax Administration, Japan, for her valuable help.

REFERENCES

1. United States Renal Data System. ASN 2003 USRDS presentations & posters. Infectious complications of hemodialysis, peritoneal dialysis, transplant, and chronic kidney disease patients. Epidemiology of infections in CKD patients in the medicare database. *USRDS 2003 Annual Report* (http://www.usrds.org).
2. Medzhitov R, Janeway Jr C. Innate immunity. *N Engl J Med* 2000; **343**: 338–344.
3. Chow JC, Young DW, Golenbock DT *et al.* Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 1999; **274**: 10689–10692.
4. Lien E, Means TK, Heine H *et al.* Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J Clin Invest* 2000; **105**: 497–504.
5. Bienvenu J, Monneret G, Fabien N, Revillard JP. The clinical usefulness of the measurement of cytokines. *Clin Chem Lab Med* 2000; **38**: 267–285.
6. Jung T, Schauer U, Heusser C *et al.* Detection of intracellular cytokines by flow cytometry. *Immunol Methods* 1993; **159**: 197–207.
7. Picker LJ, Singh MK, Zdraveski Z *et al.* Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 1995; **86**: 1408–1419.
8. Ando M, Shibuya A, Yasuda M *et al.* Innate cellular response to *ex-vivo* stimuli is impaired in patients on continuous ambulatory peritoneal dialysis. *Nephrol Dial Transplant* 2005; **20**: 2497–2504.
9. Tamandl D, Bahrami M, Wessner B *et al.* Modulation of toll-like receptor 4 expression on human monocytes by tumor necrosis factor and interleukin-6: tumor necrosis factor evokes lipopolysaccharide hyporesponsiveness, whereas interleukin-6 enhances lipopolysaccharide activity. *Shock* 2003; **20**: 224–229.
10. Netea M, van der Meer JM, van Deuren M, Kullberg BJ. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol* 2003; **24**: 254–258.
11. Riedemann NC, Guo R-F, Ward PA. Novel strategies for the treatment of sepsis. *Nat Med* 2003; **9**: 517–524.
12. Wolfgang E, Kremer J-P, Kenny J *et al.* Downregulation of proinflammatory cytokine release in whole blood from septic patients. *Blood* 1995; **85**: 1341–1347.
13. Flach R, Majetschak M, Heukamp T *et al.* Relation of *ex vivo* stimulated blood cytokine synthesis to post-traumatic sepsis. *Cytokine* 1999; **11**: 173–178.
14. Dissel JT, van Langevelde P, Westendorp RGJ *et al.* Anti-inflammatory cytokine profile and mortality in febrile patients. *Lancet* 1998; **351**: 950–953.
15. Gogos CA, Drosou E, Basseris HP, Skontelis A. Pro- versus anti-inflammatory cytokine profile in patients with sever sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis* 2000; **181**: 176–180.
16. Tabeta K, Yamazaki K, Akashi S *et al.* Toll-like receptors confer responsiveness to lipopolysaccharide from Porphyromonas gingivalis in human gingival fibroblasts. *Infect Immun* 2000; **68**: 3731–3735.
17. Akashi S, Shimizu R, Ogata H *et al.* Cell surface expression and lipopolysaccharide signaling via the Toll-like receptor MD2 complex on mouse peritoneal macrophages. *J Immunol* 2000; **164**: 3471–3475.
18. Openshaw P, Murphy EE, Hosken NA *et al.* Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med* 1995; **182**: 1357–1367.