

*Original Article*

## **Quantitative flow cytometry shows activation of the TNF- $\alpha$ system but not of the IL-2 system at the single cell level in renal replacement therapy**

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### **Abstract**

**Background.** Immunological dysfunction in patients on haemodialysis may be related to imbalanced cytokine systems, such as tumour necrosis factor (TNF)- $\alpha$  and interleukin (IL)-2. Despite activation of these systems, haemodialysis patients show high susceptibility for infections and malignancies, and have a poor immunological reaction to T-cell-dependent antigens, like hepatitis B vaccination. In this study we have determined the activation status of the two different cytokine systems, at the single cell level, using quantitative flow cytometry.

**Methods.** Using fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies directed against TNF-R2 (CD120b), IL-2R $\alpha$  (CD25) and IL-2R $\beta$  (CD122), we measured the expression of these receptors at the single cell level in order to determine the level of activation of monocytes and T-lymphocytes.

**Results.** Significantly higher expression of the TNF- $\alpha$  receptor, TNF-R2, was present on both monocytes and T-lymphocytes in patients on renal replacement therapy (RRT) compared with pre-dialysis chronic renal failure (CRF) patients and controls, indicating activation of the TNF- $\alpha$  system. In contrast, IL-2R expression was comparable in all groups studied, which may reflect a non-activated state of the IL-2 system.

**Conclusions.** The present study illustrates an activated state of the TNF- $\alpha$  system in patients on RRT, at the single cell level, while the IL-2 system seems to be unaffected. These findings support the hypothesis that the interaction between the TNF- $\alpha$  and IL-2 cytokine systems is disturbed.

**Keywords:** activation; chronic renal failure; IL-2; quantitative flow cytometry; renal replacement therapy; TNF- $\alpha$

### **Introduction**

Patients with chronic renal failure (CRF) and on renal replacement therapy (RRT) suffer from a high susceptibility to infections and show a higher incidence of malignancies compared with healthy controls [1]. This immune incompetence may be due to imbalanced defence mechanisms in which cytokines derived from antigen-presenting cells (APC) and T-cells play a central role. Kimmel *et al.* reported that the immunological dysfunction in patients on haemodialysis (HD) is related to overall survival [2]. In patients with ESRF, on peritoneal dialysis (CAPD) or haemodialysis (HD), various cytokine systems are affected [3–5]. TNF- $\alpha$  is a pro-inflammatory cytokine which induces expression of MHC class I molecules, and activates the production of enzymes and adhesion molecules. It can induce programmed cell-death and is needed for T-cell proliferation. It is mainly produced by activated monocytes and macrophages and, to a lesser extent, by lymphocytes [6]; therefore it provides a pivotal role in the function of APC, whereas IL-2 is regarded as a central T-cell cytokine that promotes expansion of T-cells, augments the cytolytic activity of NK cells, and is involved in programmed cell death of activated T-cells and in the synthesis of immune globulins by B-cells [7]. Active TNF- $\alpha$  is unstable, and inadequate collection and processing of blood samples may result in undetectable circulating cytokines, while also the IL-2 protein measurements are influenced by mode of collection [8]. Measurements of free plasma TNF- $\alpha$  can be strongly influenced by the above-mentioned factors. In addition, variable results of TNF protein measurements using commercially available ELISA kits complicate comparisons between studies [9,10].

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**Table 1.** Patient characteristics

	Controls	CRF	CAPD	HD
Number	9	11	8	12
Male : female	4 : 5	3 : 8	7 : 1	6 : 6
Age in years (mean $\pm$ SD)	35.6 $\pm$ 8.6	45.6 $\pm$ 19	44.5 $\pm$ 14	57.8 $\pm$ 15
Race				
Caucasian	7	7	6	8
Black	0	1	0	2
Oriental	1	2	2	2
Other	1	1	0	0
Original disease				
Hypertension	0	4	3	4
Membranous glomerulonephritis	0	2	2	4
M. Wegener	0	1	0	1
Focal segmental glomerulosclerosis	0	1	0	1
Adult polycystic kidney disease	0	0	2	2
IgA nephropathy	0	1	0	0
Amyloidosis	0	1	0	0
Unknown	0	1	1	1
Medication				
Ca <sup>2+</sup> -entry blockers	0	4	2	3
ACE inhibitors	0	1	4	8
$\beta$ -blockers	0	8	4	4
Erythropoietine	0	2	6	10

Age in years.  $P < 0.01$  for HD vs controls using ANOVA. Ca<sup>2+</sup>-entry blockers = calcium entry blockers: nifedipine and isradipine.  $\beta$ -blockers =  $\beta$  receptor antagonists: atenolol and metoprololtartrate. Erythropoietine = human recombinant erythropoietine subcutaneously.

In contrast, measurements of the expression of membrane-bound activation markers by flow cytometry provide an elegant, reproducible and sensitive tool to determine the activated state of cytokine systems at the single cell level. In this study, we used flow cytometry to quantitate the expression of activation markers of both the TNF- $\alpha$  and IL-2 system: the TNF-R2 (CD120b) on lymphocytes and monocytes, and the IL-2R $\alpha$  (CD25) and IL-2R $\beta$  (CD122) chains on  $\alpha/\beta$  T-cell receptor (TCR)-positive T-cells.

## Subjects and methods

### Patients

In 11 patients (three males, eight females; mean age 45.6  $\pm$  19 years) with pre-dialysis CRF, eight (seven males, one female; mean age 44.5  $\pm$  14 years) on CAPD, and 12 (six males, six females; mean age 57.8  $\pm$  15 years) on HD, the activation markers of the TNF- $\alpha$  and IL-2 system were determined on peripheral T-lymphocytes and monocytes using quantitative flow cytometry. Mean serum creatinine was 552  $\mu$ mol/l (CRF), 1031  $\mu$ mol/l (CAPD) and 995  $\mu$ mol/l (HD). The patients with pre-dialysis CRF had a mean creatinine clearance of 13  $\pm$  2.5 ml/min. In patients on RRT, the Kt/V was routinely determined using specific software (Baxter), in which the volume (V) is calculated according to the formula of Watson (in relation to length, gender, body weight and age). In HD patients and in those on CAPD, a Kt/V of 1.3  $\pm$  0.2 and 1.5  $\pm$  0.2 was obtained, respectively. Mean time on dialysis was 27.4  $\pm$  8.3 months for CAPD patients and 26.1  $\pm$  5.5 months for HD patients. The dialyser membranes used in the study were Polysulphone (F60 Fresenius, AG, Bad Homburg, Germany) and Hemophane (MA-12H, Kawasaki Laboratory Inc., Minamiohi, Tokyo, Japan).

Both dialysers are known for their better bio-compatibility than cuprophane membranes [11]. Previously we have shown that patients dialysed with either of the two membranes showed no differences in activation parameters of the TNF- $\alpha$  system [12]. The HD patients were on bicarbonate dialysate. The dialysate is routinely cultured and no periods of contamination were found ( $< 10^3$  micro organisms per millilitre). The patients on CAPD used the Baxter twinbag system (2 l, four times daily). Four weeks before and during, and at least 4 weeks after blood collection the patients on CAPD showed no signs of peritonitis. Blood samples were collected during the infection-free period. In HD patients, blood samples were collected before the start of the haemodialysis procedure. At the time of blood sample collection no patients were known to have a malignancy. In CRF and CAPD patients, blood samples were collected during routine outpatient visits. Causes of renal failure were hypertension ( $n = 10$ ), membranous glomerulonephritis ( $n = 8$ ), Wegener's granulomatosis ( $n = 2$ ), focal segmental glomerulosclerosis ( $n = 2$ ), polycystic kidney disease ( $n = 4$ ), IgA nephropathy ( $n = 1$ ), amyloidosis ( $n = 1$ ) and unknown ( $n = 3$ ). Most patients used anti-hypertensive drugs, i.e. calcium-entry blockers, angiotensin-converting-enzyme (ACE)-inhibitors or  $\beta$ -blockers. Subcutaneous recombinant erythropoietine treatment was started in all patients with haemoglobin levels of  $< 6$  mmol/l (9.7 g/dl). None of the patients used corticosteroids. Amongst the patients and controls there were no diabetics. All data were compared with those of nine healthy controls, who were not on medication (four males, five females; mean age 35.6 years; mean creatinine  $< 100$   $\mu$ mol/l). Patient characteristics are summarized in Table 1.

### Sample preparation and flow cytometric analysis

Blood samples were collected in pyrogen-free tubes, containing EDTA in a final concentration of 1 mg/ml. Whole blood EDTA samples were monitored for the presence of the

immune-competent cells: monocytes (CD14+) and lymphocytes ( $\alpha/\beta$ -TCR-positive). Surface activation markers were analysed by two-colour flow cytometry after staining with monoclonal antibodies directed against CD14 (Immunotech, Marseille, France) as a marker for the monocytes, and WT31 (Becton Dickinson, Mountain View, CA, USA) as a marker for the  $\alpha/\beta$  chain of the TCR. In these subsets, CD25 (IL-2R $\alpha$ , Becton Dickinson), CD122 (IL-2R $\beta$ , Becton Dickinson) and CD120b (TNF-R2, Immunotech) were monitored. The antibodies, except CD120b, were directly conjugated to fluorescein isothiocyanate or phycoerythrin (PE). For CD120b we used a two-step staining. After the first step with CD120b, cells were incubated with F(ab)<sub>2</sub> goat-anti-Rat IgG PE. The staining procedure was performed by incubating 15  $\mu$ l of 1/100 diluted CD120b antibody with 100  $\mu$ l blood (30 min at 4°C). After washing in Hanks Balanced Salt Solution (HBSS, Gibco-BRL, Paisly, UK) with 0.1% bovine serum albumin (BSA, Sigma, St Louis, MO, USA) and 0.01% sodium azide (Merck, Darmstadt, Germany), the red blood cells were lysed by FACS Lysing Solution (Becton Dickinson). Samples were centrifuged and washed in Cell Pack (TOA, Hamburg, Germany). Flow cytometric analysis was performed on FACScan flow cytometer using Cell Quest software (Becton Dickinson). From each tube, 10 000 events ( $\alpha/\beta$ -TCR-positive, CD14+) in the gate were measured. In order to compare the measurements in time, the flow cytometer was calibrated using specific calibration beads (Calibration Beads Quantum 1000, Flow cytometry Standards Corp. San Jose, Puerto Rico, USA). Each bead contains a known amount of fluorochrome. The intensity of the fluorescence is converted to a standard curve using Quick Cal program for Quantum Beads (Becton Dickinson). The mean fluorescence is denoted as the molecular equivalent of fluorochrome, or MESF (Figure 1).

The relationship between serum creatinine and membrane receptor expression was analysed for the whole group (patients and controls,  $n=40$ ) using the Spearman rank correlation. Correlation between time on dialysis (CAPD and HD) and membrane receptor expression was also evaluated by the Spearman rank correlation test.

### Statistics

Data are recorded as mean  $\pm$  SEM, or median and range. Absolute numbers of lymphocytes and monocytes in the patient groups were compared with the unpaired student's *t* test. Differences in receptor expression between patient groups were analysed using one-way analysis of variance (ANOVA), while differences between groups, separately, were analysed using the Mann-Whitney test. The Spearman *r* correlation coefficients were used to determine the relationship between serum creatinine, time on dialysis and membrane receptor expression (IL-2R $\alpha$  (CD25), IL-2R $\beta$  (CD122) and TNF-R2 (CD120b) on monocytes and lymphocytes) and age. *P* values  $\leq 0.05$  were considered significant.

### Results

In patients with CRF (not on dialysis) and on RRT, with a mean time on dialysis of  $26.1 \pm 5.5$  months (HD) and  $27.4 \pm 8.3$  months (CAPD), we measured cytokine activation markers (CD25, CD122, CD120b) on immune-competent cells. Absolute numbers of  $\alpha/\beta$ -TCR-positive T-cells were significantly lower in HD patients than in healthy controls: ( $665 \pm$

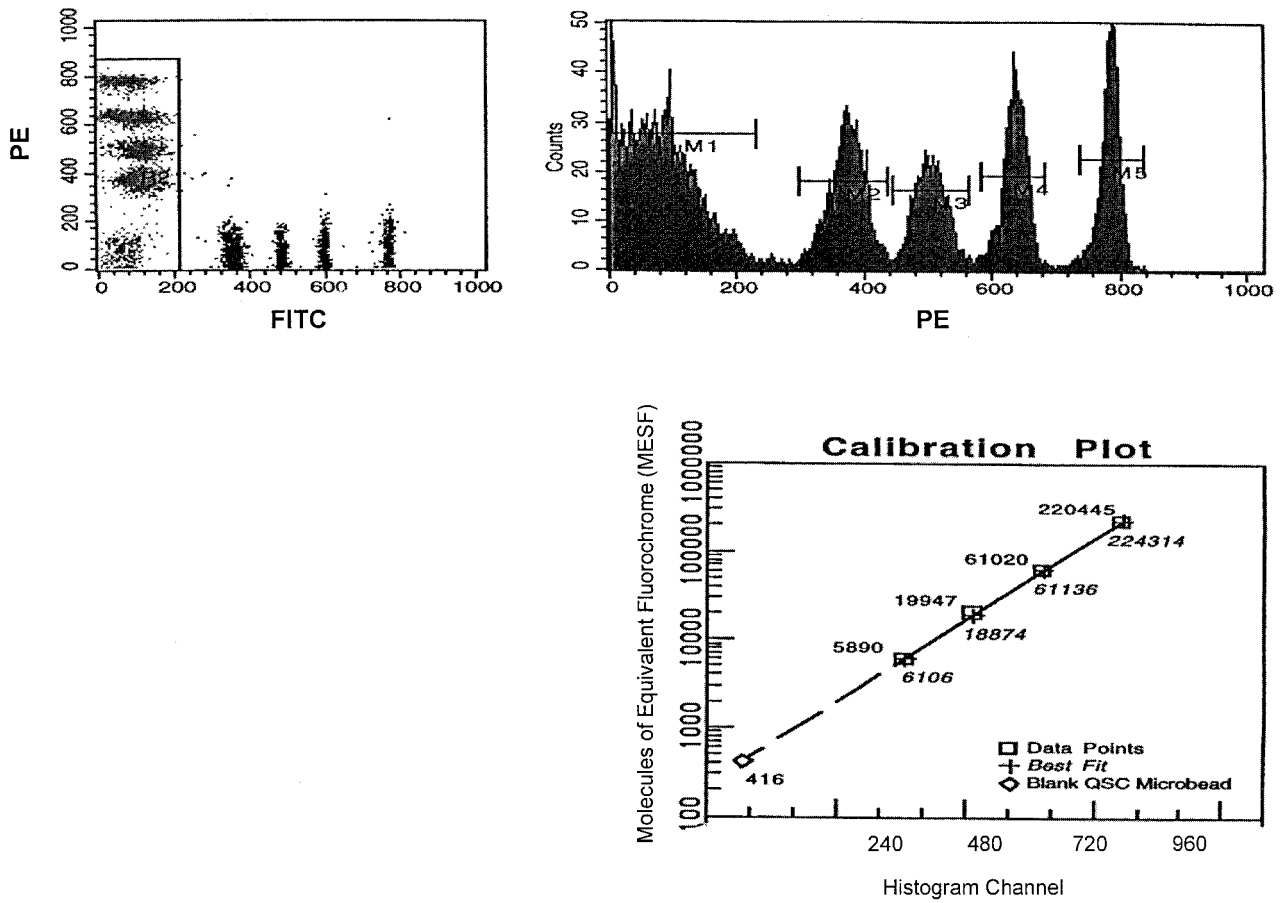
$88$  vs  $979 \pm 99$  cells/ $\mu$ l,  $P=0.02$ ). In patients on CAPD ( $673 \pm 116$  cells/ $\mu$ l) and CRF ( $738 \pm 172$  cells/ $\mu$ l), the absolute number of  $\alpha/\beta$ -TCR-positive T-cells was comparable to the number of cells found in the control group ( $P=0.07$  and  $0.22$ , respectively). The absolute number of monocytes was comparable between all groups:  $314 \pm 34$  cells/ $\mu$ l (controls),  $284 \pm 29$  cells/ $\mu$ l (CRF),  $374 \pm 59$  cells/ $\mu$ l (CAPD) and  $293 \pm 56$  cells/ $\mu$ l (HD).

Expression of the activation markers of the IL-2 system (i.e. CD25, CD122) was in the same range for patients and controls. The mean expression of IL-2R $\alpha$  (CD25) varied from  $779 \pm 132$  MESF (controls) to  $992 \pm 163$  MESF (CAPD),  $762 \pm 49$  MESF (CRF) and  $763 \pm 104$  MESF (HD) ( $P=0.51$  by ANOVA). The mean expression of IL-2R $\beta$  (CD122) ranged from  $365 \pm 14$  MESF (controls), to  $409 \pm 19$  MESF (CRF),  $401 \pm 18$  MESF (CAPD) and  $431 \pm 38$  MESF (HD) ( $P=0.40$  by ANOVA) (Figure 2). In contrast to these comparable expression levels of the activation markers of the IL-2 system on T-cells, the expression levels of the activation marker of the TNF- $\alpha$  system TNF-R2 (CD120b) were significantly higher in patients on RRT, on both monocytes and on  $\alpha/\beta$ -TCR-positive lymphocytes ( $P=0.02$  and  $0.03$ , respectively, by ANOVA). The mean TNF-R2 (CD120b) expression on lymphocytes was  $3153 \pm 508$  MESF (controls) vs  $3451 \pm 288$  MESF (CRF) ( $P=0.65$ ) CAPD:  $5670 \pm 997$  MESF ( $P=0.06$  vs controls), and in HD:  $5466 \pm 893$  MESF ( $P=0.01$  vs controls). Mean TNF-R2 (CD120b) expression on monocytes was also higher in patients on CAPD and HD, but not in CRF compared with controls. MESF was  $7285 \pm 1516$  (CAPD) vs  $2564 \pm 808$  (controls) ( $P=0.005$ ). MESF was  $10233 \pm 3531$  (HD) ( $P=0.02$  by the Mann-Whitney test) (Figure 3).

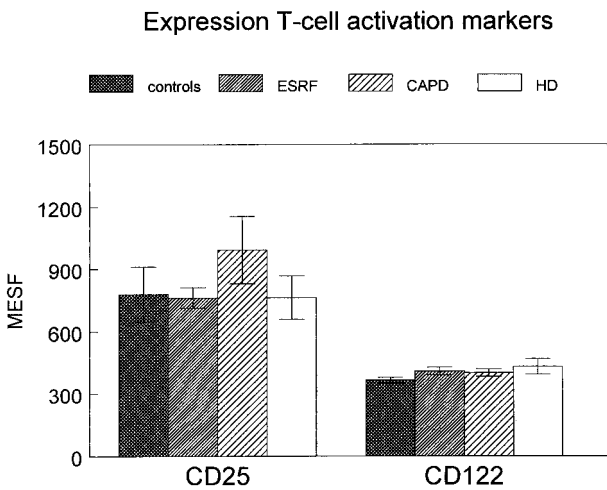
Of the studied cytokine activation markers (CD25, CD122, CD120b on lymphocytes and monocytes), only CD120b on monocytes showed a correlation with serum creatinine. The *r* coefficient for CD25 was  $0.08$  ( $P=0.62$ ), for CD122 it was  $r=0.16$  ( $P=0.31$ ), for CD120b on lymphocytes it was  $r=0.16$  ( $P=0.31$ ), and for CD120b on monocytes it was  $r=0.38$  ( $P=0.03$ ) according to the Spearman rank correlation. Except for CD120b expression on monocytes, we found no correlation between age and cytokine receptor expression: for CD25,  $r=-0.16$  ( $P=0.32$ ); for CD122:  $r=0.13$  ( $P=0.42$ ); for CD120 on lymphocytes,  $r=0.24$  ( $P=0.16$ ); and for CD120b on monocytes,  $r=0.43$  ( $P=0.01$ ), using the Spearman rank correlation. Also, time on dialysis was not correlated with the expression of the membrane receptors:  $r=0.12$ ,  $P=0.68$ , using the Spearman rank correlation.

### Discussion

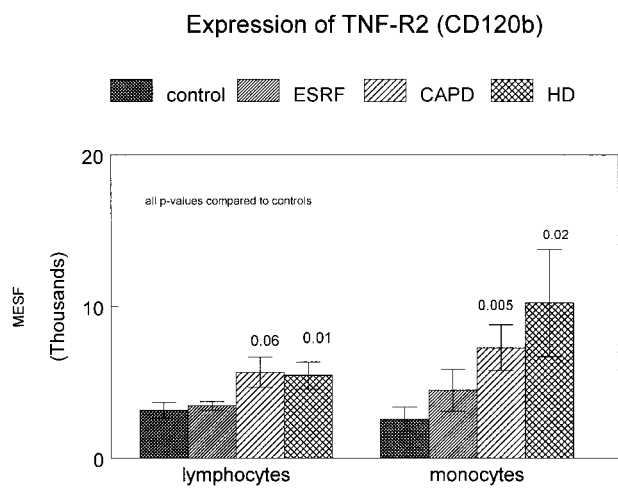
In the present study, we used quantitative flow cytometry to evaluate the expression of activation markers of the TNF- $\alpha$  and the IL-2 system in patients with CRF and on RRT. We determined the receptor



**Fig. 1.** Standard curve fluorescence intensity of calibration beads. Upper panel: flow cytometric analysis of calibration beads, each of which contains a specific amount of fluorescein. Lower panel: standard curve of fluorescence intensity constructed after flow cytometric analysis of calibration beads, using Quick Cal program for quantum beads software. The intensity of the fluorescence is depicted as MESF.



**Fig. 2.** Expression of T-cell activation markers: CD25 (IL-2R $\alpha$  chain) and CD 122 (IL-2R $\beta$  chain) in patients with end-stage renal failure (ESRF), on peritoneal dialysis (CAPD) and haemodialysis (HD), measured with quantitative flow cytometry in peripheral blood. The quantity of fluorescence is depicted as MESF. Error bars indicate the SEM.



**Fig. 3.** Expression of the TNF- $\alpha$  activation marker: CD 120b (TNF-R2) on lymphocytes and monocytes in patients with end-stage renal failure (ESRF), on peritoneal dialysis (CAPD) and haemodialysis (HD) compared with healthy controls, measured by quantitative flow cytometry. The quantity of fluorescence is depicted as MESF. Error bars indicate the SEM.

expression for TNF- $\alpha$  and IL-2 on immune-competent cells,  $\alpha/\beta$ -TCR-positive lymphocytes and the CD14+ macrophages in order to differentiate which cells, T-cell or APC, are activated. To evaluate the activation of the IL-2 system we measured the expression of IL-2R $\alpha$  (CD25) and IL-2R $\beta$  (CD122). In this study, we were predominantly interested in the expression of the TNF-R2, because TNF- $\alpha$  action directed by TNF-R2 results in proliferation of T-cells. We found reduced absolute numbers of lymphocytes in the presence of comparable absolute numbers of monocytes in patients on HD vs all other groups. It is known that activated lymphocytes are sequestered in the capillaries of the lung after blood dialyser membrane contact [13]. Deenitchina *et al.* reported that this lymphopenia was the consequence of low numbers of CD4+ T-cells [14]. We found an increased expression of the TNF-R2 on both monocytes and lymphocytes from patients on RRT, which suggests activation of the TNF- $\alpha$  system [15]. This coincides with the high levels of soluble receptors and the significantly higher levels of free plasma TNF- $\alpha$  in these patients [16]. In contrast, in patients with CRF, TNF-R2 expression was comparable to the expression measured on immune-competent cells from healthy controls. Although the groups studied are small, it appears that the TNF- $\alpha$  system is not activated in CRF patients. As the creatinine clearance in the patients with CRF in this study was 13 ml/min, which is comparable to the creatinine clearance obtained in patients on RRT, i.e. CAPD or HD, renal insufficiency alone cannot be responsible for the differences in expression of the TNF- $\alpha$  activation marker, TNF-R2, found in these patients. Therefore, we think that the increased TNF-R2 expression is not caused by renal insufficiency itself, but by RRT, i.e. CAPD or HD. Because of the comparable expression of TNF-R2 on lymphocytes and monocytes in patients on CAPD and HD, it seems that other factors than the RRT itself result in TNF-R2 expression on lymphocytes or monocytes. After TNF- $\alpha$  binding to its membrane receptor, the extracellular domains are split off and can be identified as soluble TNF-R in peripheral blood. Other causes of shedding the membrane receptor are poorly understood. The sTNF-R is predominantly metabolized by renal clearance. Due to impaired renal clearance, levels of sTNF-R show a strong positive correlation with serum creatinine and renal function [12,17]. In the present study we found no correlation between the expression of CD25, CD122 and CD120b on lymphocytes and monocytes with serum creatinine per patient group. Nor did we find a correlation between time on dialysis (CAPD or HD) and the expression of the membrane receptors. To assess a possible correlation between the expression of the TNF-R2 (CD120b) on monocytes and progressive renal insufficiency, longitudinal data are needed. Age may be another confounding factor in this study. In the literature, conflicting data are reported concerning cytokine levels and production in aged subjects. Higher [18–22] as well as lower [20, 23, 24] levels and production capacity of IL-2 and TNF- $\alpha$  in aged

subjects have been described. In the present study, the mean age of the HD patients, in contrast to the other patient groups, was significantly higher compared with the controls ( $P=0.01$  using ANOVA). There was no difference in the mean age between the patient groups. This supports the hypothesis that the increased expression of the TNF-R2 on cells of CRF, CAPD and HD patients is not the result of age, but rather the result of renal insufficiency. Moreover, no correlation was found between age and the various membrane receptor expressions studied:  $P=0.31$  (CD25),  $P=0.42$  (CD122) and  $P=0.16$  (CD120b) using the Spearman rank correlation. Various drugs are known to influence TNF- $\alpha$  levels. Recombinant erythropoietine and ACE-inhibitors may induce TNF- $\alpha$  [25, 26]. In this study, the patients were on various medication (see Table 1). No differences in the use of ACE-inhibitors or erythropoietine between the various patient groups is present. Therefore the influence of the medication is not regarded as a dominant factor influencing the results of the TNF-R2 expression.

Despite the high expression of TNF-R2 on lymphocytes, the T-cells seem not to be activated as shown by normal expression of the IL-2 activation markers on the cell membrane. The interaction between the TNF- $\alpha$  system and the T-cells appears to be inadequate, which may result in the high incidence of infection and malignancy found in HD patients. Previously, we found that the phytohaemagglutinin (PHA)-stimulated TNF- $\alpha$  production by peripheral blood mononuclear cells (PBMC) was significantly reduced in patients on HD, while PHA-stimulated IL-2 production was comparable to the production by PBMC obtained from healthy controls [16]. Others reported differences between CAPD and HD patients in the production of specific T-cell cytokines. In CAPD, the mitogen phorbol-12-myristate-13-acetate (PMA)-stimulated T-cell production of TNF- $\alpha$  and IL-2 was lower compared with the production by T-cells obtained from HD patients [27]. We suggest this impaired TNF- $\alpha$  production results from 'exhaustion' due to continuous activation of the TNF- $\alpha$  system. In HD patients, this is explained by chronic blood-membrane contact, and in CAPD patients by chronic, low-grade, subclinical intra-peritoneal inflammation [28–30]. TNF- $\alpha$  activities, conducted by TNF-R1, are responsible for signalling apoptosis, while TNF- $\alpha$  activity conducted by TNF-R2 is responsible for signalling proliferation of thymocytes and cytotoxic T-cells [15]. A distinct difference in action is, however, not overt. Monoclonal antibodies directed against TNF-R2 can partially antagonize the same TNF responses that are induced by TNF-R1. Tartaglia *et al.* reported that specific activation of TNF-R2, in the presence of PHA, results in stimulation of human T-cells [30]. The increased expression of the TNF-R2 may thus result in T-cell activation and proliferation in the presence of TNF- $\alpha$  and mitogen.

In summary, we found an activated TNF- $\alpha$  system shown by high expression of the TNF-R2 on lymphocytes and monocytes. In previous studies, increased

mRNA expression for TNF- $\alpha$  and elevated plasma TNF- $\alpha$  protein levels have already been found [12]. The IL-2 system seems not to be activated, as shown by the comparable expression of the T-cell activation markers, IL-2R $\alpha$  (CD25) and IL-2R $\beta$  (CD122), in patients and controls. The immunoregulatory activity of TNF- $\alpha$ , which results in induction of IL-2R expression and enhanced T-cell responses directed by IL-2, failed to induce T-cell activation in the patient groups we studied.

The present study shows that quantitative flow cytometry provides additional information about the activation status of cytokine systems at the single cell level, and clarifies the nature of the immune-competent cells involved in the immune responses in patients with CRF and on RRT. Our results show that interaction between APC (TNF- $\alpha$ ) and T-cells (IL-2) does not result in activation or proliferation of the T-cells in spite of the increased expression of TNF-R2 on the membrane. This may contribute to the immune deficiency in patients on intermittent haemodialysis.

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Received for publication: 28.4.00

Accepted in revised form: 9.2.01