

## Impaired T cell proliferation, increased soluble death-inducing receptors and activation-induced T cell death in patients undergoing haemodialysis

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### SUMMARY

Haemodialysis is a widespread option for end-stage renal disease (ESRD). Long-term success of dialysis is, however, limited by a high rate of serious bacterial and viral infections. We compared T cell functions in ESRD patients undergoing haemodialysis ( $n = 20$ ), or were not dialysed and received conventional medical treatment ( $n = 20$ ). Healthy volunteers ( $n = 15$ ) served as controls. The T cell phenotype was examined by immunofluorescence using fluorochrome-labelled monoclonal antibodies and FACS analysis. The concentration of soluble CD95/Fas and of tumour necrosis factor- $\alpha$  receptor type 1 (sTNFR1) in the sera was quantified by ELISA. Activation-induced programmed T cell death was triggered by anti-CD3/CD28 antibodies and measured by 7-AAD staining. All immunological tests were performed at least 1 month after dialysis initiation. T cell proliferation in response to phytohaemagglutinin or anti-CD3 monoclonal antibodies was moderately diminished in non-dialysed patients and markedly reduced in haemodialysis patients compared to healthy controls ( $P < 0.01$  and  $P < 0.001$ , respectively). In a mixed lymphocyte culture the proliferative response of T cells from dialysed patients was significantly diminished ( $P < 0.001$ ). T cells of both non-dialysed and dialysed patients have augmented CD95/Fas and CD45RO expression, increased sCD95/Fas and sTNFR1 release and spontaneously undergo apoptosis. Culture of T cells from haemodialysis patients with anti-CD3/CD28 antibodies increased the proportion of CD4<sup>+</sup> T cells committing activation-induced cell death by a mean 7.5-fold compared to T-helper cells from non-dialysed patients ( $P < 0.001$ ). Renal failure and initiation of haemodialysis results in a reduced proliferative T cell response, an aberrant state of T cell activation and heightened susceptibility of CD4<sup>+</sup> T cells to activation-induced cell death.

**Keywords** apoptosis end-stage renal failure haemodialysis T cell activation

### INTRODUCTION

Renal failure is a major public-health issue due to its permanent increase among the general population, and the escalation of the cost of the renal function replacement therapies such as dialysis and kidney transplantation. The rate of success has, however, been reduced by various complications including thromboembolic events, haemostasis disorders and arterial occlusive accidents [1–3]. Despite continuous improvement of dialysis techniques, infections have remained one of the major causes of morbidity of patients in end-stage renal disease (ESRD). Epidemiological studies within the United States indicate that in chronic haemodialysis patients, bacterial and viral infection rank second

place in mortality and morbidity, behind cardiovascular disease. Septicaemia accounts for more than 75% of mortality rate in haemodialysis ESRD patients, and an average of 7.6 bacteraemia episodes per 100 patient-years [4–6]. A European prospective cross-sectional study revealed that bacterial infections have a monthly incidence of approximately 1% [7].

The high risks of bacterial and viral infections in ESRD patients are related to an acquired immunodeficiency [5]. Uremic toxins may cause defects in cell-mediated immunity. Clinical evidence of impaired lymphocyte [8,9], granulocyte [10,11] and monocyte [5,12] functions, which progress during the development of uremic retention, has been reported. Immune defects may also occur as a direct consequence of therapy, mainly in cell-mediated immunity [13]. Although some of the infections can be attributed to catheter and dialysis device installations, the high incidence of viral and bacterial infections in particular suggests that long-term haemodialysis treatment may be associated with

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the development of defects in host immunity [7,14,15]. This has been also reported after implantation of left-ventricular assist devices (LVAD) [16,17].

The objective of the present study was to investigate T cell proliferative responses, changes in T cell phenotype, the presence of soluble death-inducing receptors and activation-induced T cell death in ESRD patients undergoing haemodialysis or conventional medical treatment (non-dialysed) in comparison to healthy humans.

## PATIENTS AND METHODS

### Patients and clinical features

Immunological investigations were carried out in 20 patients undergoing haemodialysis three times per week (chronically uremic patients; mean creatinine  $8.26 \pm 0.48$ ), in 20 non-dialysed patients (predialysis), non-catabolic renal failure patients (ESRD-chronically azotemic patients; with a mean serum creatinine value of  $6.33 \pm 0.41$  mg/dl), and in 15 apparently healthy volunteers. The study groups were age- and sex-matched and all immunological tests were performed in the dialysis group at least 1 month after initiation of dialysis. Polysulphone and cellulose acetate filters were utilized for haemofiltration. None of the renal failure patients had concurrent opportunistic or viral infections. Blood samples were acquired before scheduled dialysis treatment. Some demographic and clinical features are shown in the Table 1.

### Immunophenotype of circulating T cells

Cell counts were measured by Coulter counter analysis. Fluorochrome-labelled monoclonal antibodies (MoAbs) to CD3, CD4, CD8, CD45RO and CD95 (Becton Dickinson, McKinley, MN, USA) were utilized in two-colour immunofluorescence analyses as described previously [16]. Immunofluorescence was measured by a FACScan 500 flow cytometer (Becton Dickinson, San Jose, CA, USA).

### T cell proliferation

Whole blood samples had been anticoagulated with edetic acid, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation [17]. PBMC were resuspended in culture medium (Roswell Park Memorial Institute Medium), and 200  $\mu$ l medium containing  $10^5$  cells was added per well of 96-well flat-bottomed tissue culture plates. For blastogenesis assays cells were stimulated with phytohaemagglutinin (PHA;

7  $\mu$ g/ml; Sigma, St Louis, MO, USA) or MoAb to CD3 (10  $\mu$ g/ml) for 48 h at 37°C. For mixed lymphocyte culture (MLC) assays,  $5 \times 10^5$  responder cells (isolated from either healthy humans, predialysis and dialysis patients) were added to irradiated (25 Gy) stimulator cells ( $5 \times 10^5$  cells) (obtained from controls) or to the medium only, and cultured for 5 days at 37°C. Cells were pulsed for 16 h with [<sup>3</sup>H]thymidine ( $3.7 \times 10^4$  Bq/well) after 48 h in the blastogenesis assays, and after 5 days in the MLC. Cells were harvested and [<sup>3</sup>H]thymidine uptake was measured in a liquid scintillation counter.

### Quantification of soluble CD95 (sCD95)

Serum samples were obtained from study populations and kept frozen. Circulating serum levels of sCD95 were measured in a commercial enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies against human CD95 (Cytoscreen, BioSource International, Inc., Camarillo, CA, USA), as described previously [18]. The sensitivity limit of the ELISA was 20 pg/ml. The amount of protein in each serum sample was calculated according to a standard curve of optical density values constructed for known levels of sCD95 protein.

### Quantification of soluble tumour necrosis factor- $\alpha$ receptor type 1 (sTNFR1)

Serum samples were obtained from study populations and kept frozen. Circulating serum levels of sTNFR1 (TNFR p55, CD120a) were measured by ELISA [19], using polyclonal antibodies against human TNFR1 (R&D Systems Inc, McKinley Place, MN, USA). The sensitivity limit of the ELISA was 3 pg/ml. The amount of protein in each sample was calculated according to a standard curve of optical density values constructed for known levels of sTNFR1 protein.

### T cell apoptosis in vivo

A flow cytometry apoptosis detection kit (Becton Dickinson Systems, McKinley, MN, USA) was used to identify programmed cell death. PBMC ( $3 \times 10^5$ ) were triple-stained with phycoerythrin-conjugated MoAb to CD3, 10  $\mu$ l fluorescein isothiocyanate-labelled annexin V (R&D Systems, Minneapolis, MN, USA) to detect phosphatidylserine expression on cells during early apoptotic phases and 7-aminoactinomycin (7-AAD) to exclude dead cells [20,21]. The samples were analysed on a FACStar 500.

### Activation-induced cell death

The amount of  $3 \times 10^6$  PBMC from dialysis, predialysis and healthy humans (each group,  $n = 6$ ) were placed on 48-well plates and cultured with MoAb to CD3/CD28 (10  $\mu$ g/ml) or isotype control antibodies for 18 h at 37°C. Resting or stimulated T cells were stained simultaneously with fluorochrome-conjugated anti-CD4 MoAb or antiannexin V/7-AAD [21], and subjected to flow cytometry analysis.

### Statistical analysis

Continuous variables such as proliferative responses to various stimuli, expression of CD95 and CD45RO, serum level of sCD95 and sTNFR, or annexin V binding were analysed by Student's *t*-test. A value of  $P < 0.05$  was considered to be statistically significant.

**Table 1.** Demographic and clinical features

	Uremic patients ( $n = 20$ )	Azotemic patients ( $n = 20$ )
Age years, median (range)	58 (31–85)	53 (33–78)
Gender; male:female	11: 9	13: 7
Serum-creatinine; mg/100 ml	$8.26 \pm 0.48^*$	$6.33 \pm 0.41^*$
Kt/V	$1.46 \pm 0.06$	–
Creatinine-clearance; ml/min	–	$11.6 \pm 1.03$
WBC; G/l	$6.17 \pm 0.42$	$6.85 \pm 0.4$
C-reactive protein; mg/100 ml	$0.71 \pm 0.17$	$0.43 \pm 0.13$

Data except age and gender are expressed as mean  $\pm$  s.e.m. Statistical significance: \* $P < 0.01$  when compared to each other.

**Table 2.** Changes in the number and the phenotype of T lymphocytes

Lymphocytes	Controls (n = 15)	Predialysis (n = 20)	Dialysis (n = 20)
T cells (%)	29.4 ± 1.7	21.3 ± 1.2*	19.7 ± 1.4*
CD3 <sup>+</sup> /μl	1259 ± 118	1079 ± 95*	917 ± 85**
CD4 <sup>+</sup> /μl	727 ± 87	702 ± 63	521 ± 46**
CD8 <sup>+</sup> /μl	520 ± 53	383 ± 41*	299 ± 29**
CD4:CD8 ratio, mean	1.42 ± 0.09	2.02 ± 0.19*	1.93 ± 0.19*

Peripheral T cells and their subsets were identified by immunofluorescence and FACS analysis. Data represent the mean ± s.e. Statistically significant differences between T cells from dialysis and predialysis patients *versus* controls: \* $P < 0.01$ ; \*\* $P < 0.001$ . The value of CD4:CD8 ratio from each patient was used to calculate the mean.

## RESULTS

### Immunophenotypic analysis of T cells

As shown in Table 2, the percentage of circulating T lymphocytes was significantly lower in dialysis as well as in predialysis patients ( $P < 0.01$ ) compared with healthy control volunteers (29.4 ± 1.7; 21.3 ± 1.2; 19.7 ± 1.4, respectively). Among T cell subsets the mean number of circulating CD4<sup>+</sup> T cells was significantly lower in dialysis (521 ± 46) than in the predialysis group (702 ± 63) of patients ( $P < 0.001$ ), or healthy controls (727 ± 87). In both groups of dialysed and non-dialysed ESRD patients the mean counts of CD8<sup>+</sup> T cells was significantly reduced.

### T cell proliferation

The results presented in Fig. 1a demonstrate that the proliferative capacity of T cells from ESRD-predialysis and dialysis patients in response to PHA were significantly reduced in comparison to controls ( $P < 0.01$  and  $P < 0.001$ , respectively). As can be seen from Fig. 1b, after activation of T cells with anti-CD3 MoAb, the mean stimulation index (SI) of T cells from dialysed (40.9 ± 14.5) and non-dialysed (125.9 ± 19.7) patients were markedly reduced ( $P < 0.01$  and  $P < 0.001$ , respectively) *versus* healthy controls (168.9 ± 23.9). After allogeneic MLC, the SI of T cells only from dialysed patients was significantly diminished ( $P < 0.001$ ) as compared to the control group (Fig. 1c).

### Expression of CD95 and CD45RO on peripheral T cells

As shown in Fig. 2a, the expression of CD95 (Fas, Apo1), a molecule associated with a pathway of cellular apoptosis, was increased to a similar degree in CD3<sup>+</sup> T cells from the non-dialysed (77.4 ± 1.6) and dialysed (73.3 ± 1.2) patients compared with healthy control volunteers (21.8 ± 1.9). Correspondingly, as can be seen from Fig. 2b, the percentage of cells expressing CD45RO, a marker for memory T cells, was higher on CD3<sup>+</sup> T cells from both non-dialysed and dialysed patients than that in controls (79.2 ± 1.7, 72.9 ± 1.7 and 40 ± 6, respectively).

### Soluble CD95 and sTNFR1

Translocation of phosphatidylserine, an early component of T cell apoptosis, occurs after ligation of CD95 receptor/Fas ligand, and this may lead to T cell activation, followed by antigen shedding and cell suicide. Therefore, we measured circulating levels of sCD95. As shown in Fig. 3a, the mean serum levels of sCD95

were significantly higher in both dialysed and non-dialysed patients *versus* controls (10.05 ± 1.3; 4.80 ± 0.26 and 2.90 ± 0.1, respectively, all  $P < 0.001$ ).

Immune activation of lymphocytes and induction of apoptotic pathways can be initiated by TNF and results in TNFR1 shedding. To investigate the susceptibility of T cells to undergo increased activation-induced cell death (AICD) in dialysis *versus* non-dialysed patients, we measured circulating levels of sTNFR1. As can be seen from Fig. 3b the mean concentrations of sTNFR1 were significantly higher in both dialysed and non-dialysed patients *versus* controls (20.9 ± 2.2; 12.4 ± 1.8; and 0.53 ± 0.05, respectively, all  $P < 0.001$ ).

### Spontaneous T cell apoptosis in vivo

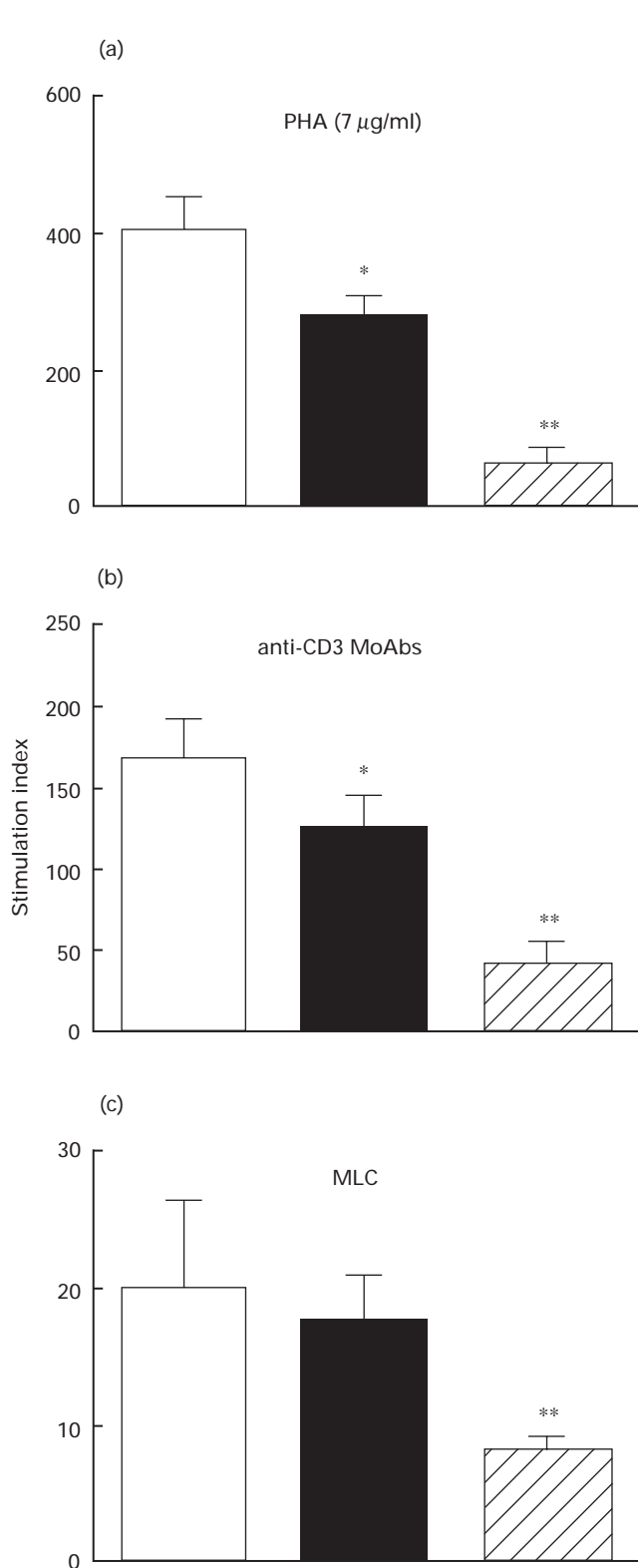
In further experiments we addressed the question of whether the elevated state of T cell activation is associated with increased percentage of peripheral T cells undergoing apoptosis. The results depicted in Fig. 4 show that CD3<sup>+</sup> T cells from non-dialysed and dialysed patients had higher levels of annexin V binding than CD3<sup>+</sup> T cells from controls (63.7 ± 4.25; 65.1 ± 3.85; 18.2 ± 1.0, respectively, all  $P < 0.001$ ). *In vivo* dead cell exclusion was performed in all samples. Each group contained less than 0.5% 7-AAD positive cells.

### Susceptibility to activation-induced cell death after T cell receptor involvement

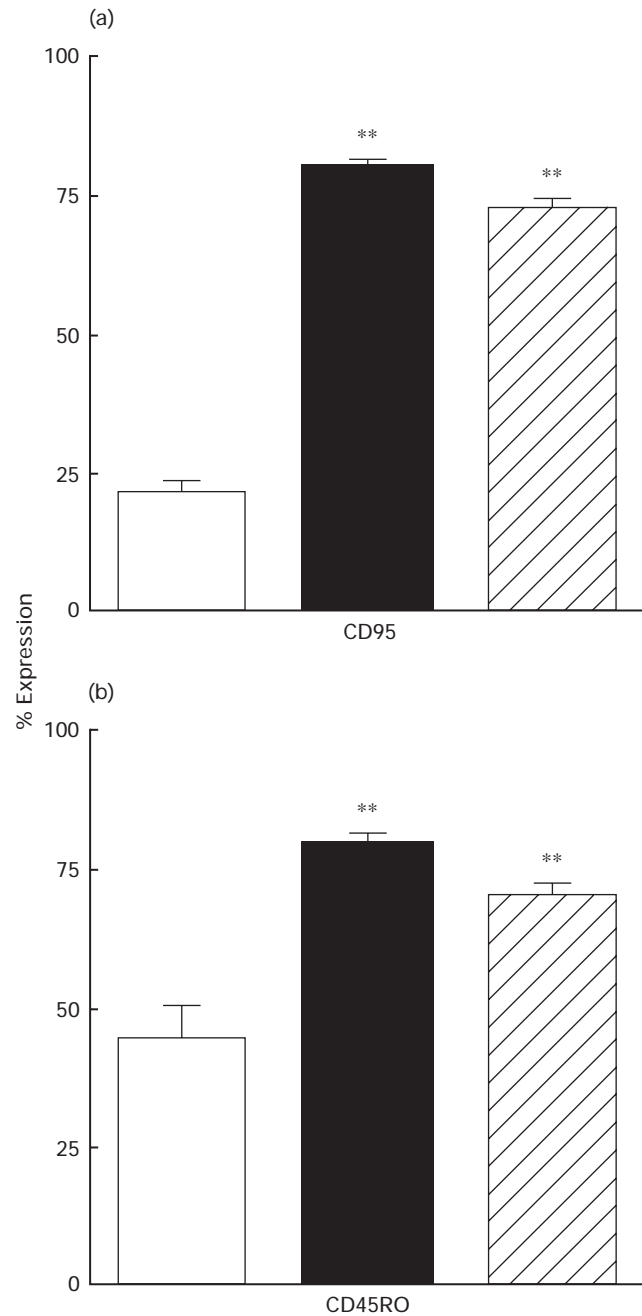
Since pre-activated T cells, which express CD95, are susceptible to activation-induced apoptosis after stimulation via the T cell receptor/CD3 complex, we investigated whether the observed defects in the proliferative responses of T cells in ESRD patients were related to activation-induced programmed cell death. As shown in Fig. 5, in a representative example of flow cytometry analysis 2.5% of resting CD4<sup>+</sup> T cells from a non-dialysed patient expressed 7-ADD after 18 h of culture in medium with IgG control, and 3.5% after stimulation with anti-CD4/CD28 antibodies. When T cells from a dialysis patient were cultured for 18 h with IgG or anti-CD3/CD28 antibodies 2.6% and 8.1% of CD4<sup>+</sup> cells were stained by 7-ADD, respectively (Fig. 5).

## DISCUSSION

An aberrant state of T cell activation involving the CD95/Fas pathway and activation-induced CD4<sup>+</sup> T cell death was found in ESRD patients. Defects in the cellular and humoral immune



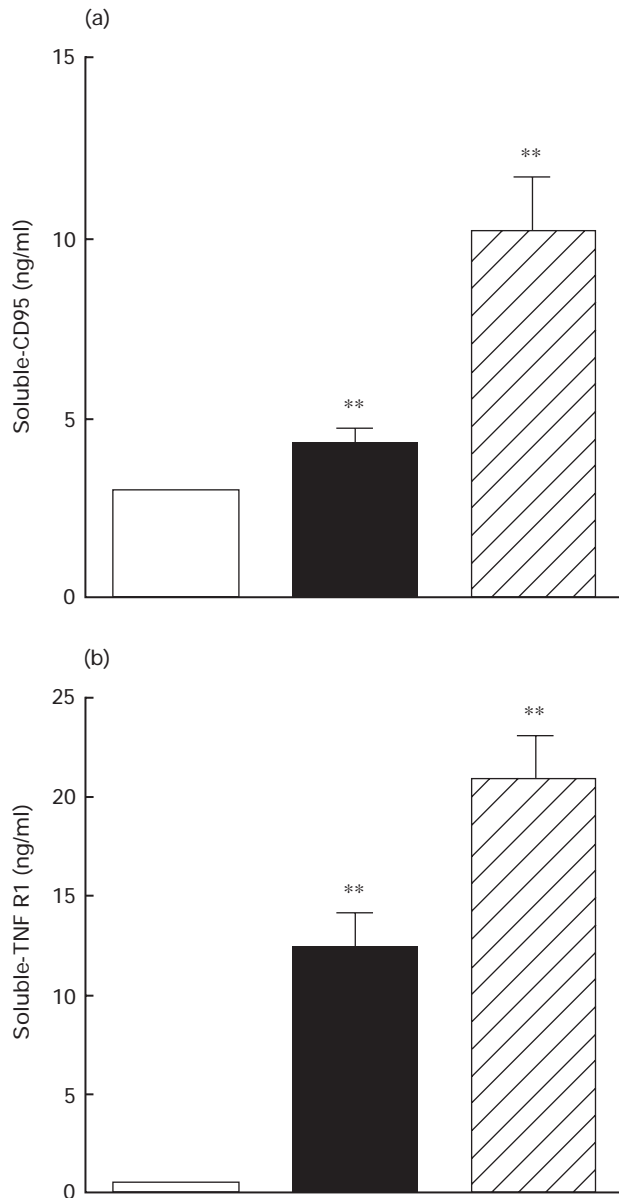
**Fig. 1.** T cell proliferative responses. PBMC from 15 healthy volunteers, 20 non-dialysed ESRD patients and 20 dialysed patients were stimulated with phytohaemagglutinin (PHA; 7  $\mu\text{g/ml}$ ), anti-CD3 mAbs (10  $\mu\text{g/ml}$ ) or in a mixed lymphocyte culture (MLC). [ $^3\text{H}$ ]thymidine incorporation was measured, and the results are presented as mean of stimulation index  $\pm$  s.e. Statistical significance: \* $P < 0.01$ ; \*\* $P < 0.001$ .  $\square$ , Healthy controls;  $\blacksquare$ , ESRD-predialysis;  $\square$  (hatched), dialysis.



**Fig. 2.** Changes in the phenotype of T cells. Expression of CD95, CD45RO on peripheral (CD3<sup>+</sup>) T cells from 15 healthy volunteers, 20 non-dialysed ESRD patients and 20 dialysed patients. Data from FACS analysis are percentage (mean  $\pm$  s.e.) increase. Statistical significance: \*\* $P < 0.001$ .  $\square$ , Healthy controls;  $\blacksquare$ , ESRD-predialysis;  $\square$  (hatched), dialysis.

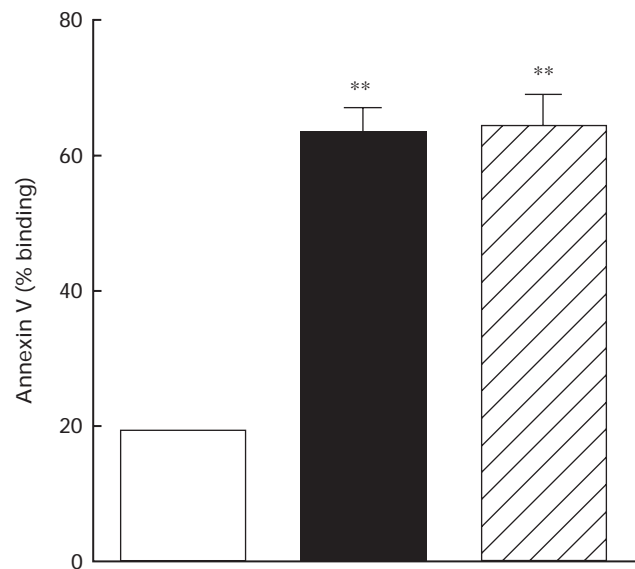
response may be responsible for the higher incidence of infection in haemodialysis patients, a major cause of increased morbidity and mortality rate [4–7]. In these patients, a functional T and B cell impairment may be accompanied by alloreactive T cell recognition of processed autoantigens, leading to production of autoantibodies specific for HLA molecules [22,23], cardiolipin, molondialdehyde-LDL,  $\beta_2\text{GPI}$  and endothelial cells [24–26].

It has been shown previously that in ESRD patients T cells and macrophages, as well as other antigen-presenting cells (APC), are



**Fig. 3.** Serum levels of soluble CD95 and tumour necrosis factor- $\alpha$  receptor type I (TNFR1). Serum samples were obtained from 15 healthy volunteers, 20 non-dialysed ESRD patients and 20 dialysed patients. The concentrations of sCD95 and sTNFR1 were measured by ELISA. The data are presented as mean (ng/ml  $\pm$  s.e.). Statistical significance:  $***P < 0.001$ .  $\square$ , Healthy controls;  $\blacksquare$ , ESRD-predialysis;  $\text{▨}$ , dialysis.

functionally activated, as defined by alteration of cytokine profile [27,28] and increased production of coagulation factors [28–30]. Furthermore, in patients undergoing haemodialysis or chronic ambulatory peritoneal dialysis, the macrophage biological functions such as adhesion, phagocytosis and killing and their capacity to present antigen to T-helper cells may be influenced markedly [5,31]. In ESRD patients, elevated sCD40 levels correlates strongly with creatinine concentration [32]. In addition, sCD23 levels correlated closely with the production of TNF- $\alpha$  and interleukin-6 [33]. These results indicate that patients with chronic renal failure often present an immunodeficiency state paradoxically exacerbated by haemodialysis with signs of B cell and

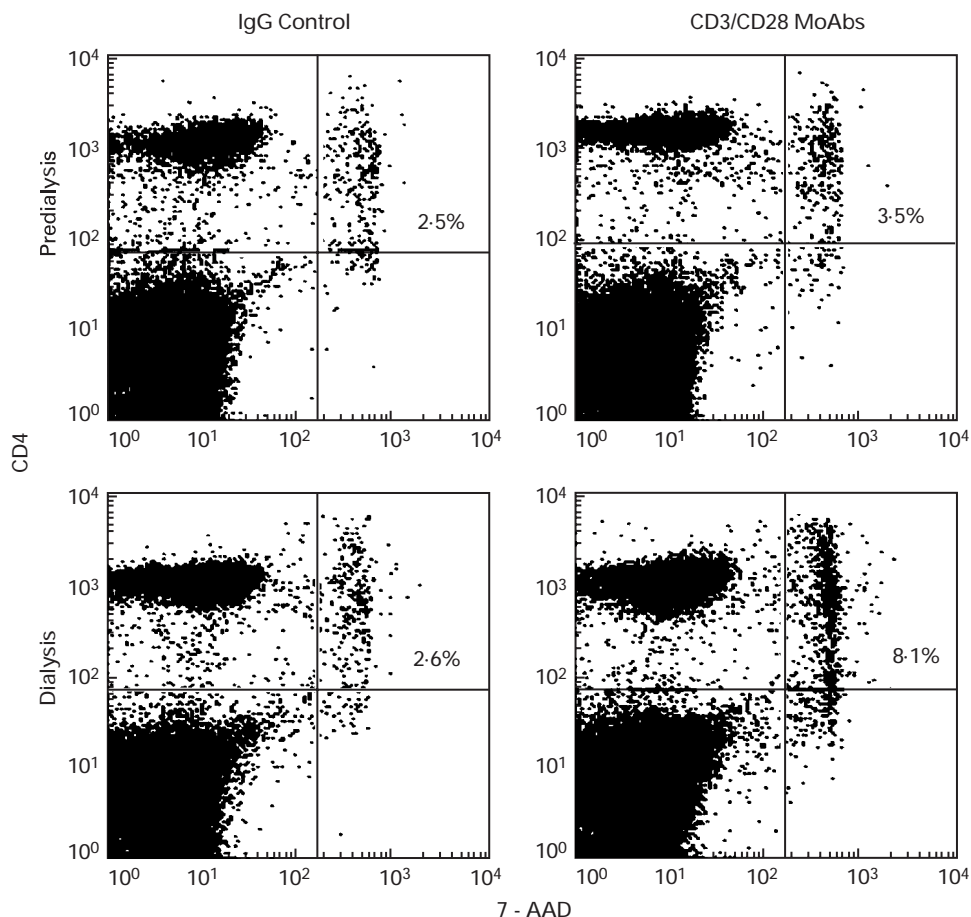


**Fig. 4.** Spontaneous T cell apoptosis. PBMC from 15 healthy volunteers, 20 non-dialysed ESRD patients and 20 dialysed patients were stained for annexin V binding. Results from FACS analysis are presented as percentage (mean  $\pm$  s.e.) of cell binding annexin V. Statistical significance:  $***P < 0.001$ .  $\square$ , Healthy controls;  $\blacksquare$ , ESRD-predialysis;  $\text{▨}$ , dialysis.

monocyte activation. Since triggering via the T cell antigen receptor and the CD3 complex increases expression of CD95/Fas and its ligand (CD95L/FasL), subsequent T cell exposure to antigenic stimulation may result in activation-induced cell death, rather than the appropriate cell proliferative response, most probably because of interactions between CD95 and newly expressed CD95L.

The co-existence of defects in T cell-mediated immunity and prominent B-cell hyperreactivity in renal failure and dialysis patients is similar to HIV-1 infection [34]. Selective depletion of CD4<sup>+</sup> T cells and immune dysfunction in AIDS patients accompanies a progressive increases in viral burden within APC, such as macrophages and dendritic cells [35,36]. One proposed mechanism to explain these findings is the inappropriate induction of apoptotic Th cell death, resulting from HIV-1-mediated interactions between CD95 and CD95L [37,38], suggesting the involvement of excessive T cell costimulation by virus-infected APC. However, by contrast with renal failure and dialysis initiation, the severe immune dysfunction associated with HIV-1 infections is multi-factorial, and apoptosis may be one of many immune defects induced by virus infection.

This paper demonstrates that patients undergoing haemodialysis present similar immune alterations compared to patients receiving a left ventricular assist device (LVAD) as a bridge to transplantation. LVAD recipients' T cells exhibit heightened susceptibility to AICD augmented by the CD95 pathway [16,17]. In both entities prolonged interaction between immunocompetent blood cell and polymers is prevalent. As LVAD patients are not under any immunosuppression we deduced previously that the chronically exposure to antigen, e.g. LVAD surface (polyurethane) or other polymers, such as silicone, PTFE or Dacron, is triggering a state of susceptibility towards programmed cell death [39,40]. We were able to demonstrate that commonly utilized polymers are



**Fig. 5.** Antibody-induced T cell death. PBMC from a dialysis patient were cultured for 18 h with IgG control or anti-CD3/CD28 antibodies, and stained with fluorochrome-conjugated anti-CD4 and anti-7-AAD monoclonal antibodies. Data from one representative experiment show the percentage flow cytometry analysis of CD4<sup>+</sup> T cells that underwent activation-induced cell death (7-AAD staining). The percentage of cells stained by 7-AAD dye is indicated in the upper right quadrant.

able to act as superantigens, hence inducing nuclear factor of activated T cells (NFAT) and selective FasL/CD95L enhancement, as detected by immunoblotting analysis [39]. This observation is supported further by the induction of caspase 8 and 3 in naive PBMC exposed to biomaterials, indicating that a chemical compound leached from biopolymers initiates a proapoptotic pathway [40]. The increased pronicity of PBMCs from dialysis patients to undergo AICD is in line with recent data, demonstrating the presence of plastic softeners in dialysis patients and their immunosuppressive potential *in vitro* [41,42]. Our findings warrant a separate longitudinal study to address the relationship between CD95 and TNFR1 mediated T cell apoptosis, reduction in CD4<sup>+</sup> T cell number and infectious complications in the cohort study.

Septicaemia remains a frequent cause of death of ESRD patients [4–7]. The results of our findings have two important implications. First, they identify an immunological activation pathway of T cells and suggest that CD95 and TNFR1 involvement relates to increased apoptosis in lymphocytes of ESRD patients. Secondly, this provides a mechanism to account for a progressive reduction in T cell counts, and suggests a genesis by which individuals become susceptible to infections. The data are consistent with the idea of reduced number of circulating T

cells in dialysed and non-dialysed patients, shedding of CD95 and TNFR1 and increased T cell susceptibility to undergo activation-induced cell death triggered by augmentation of the T cell receptor and CD3 complex.

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