

# Increased Expression of Erythropoiesis Inhibiting Cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-10, and IL-13) by T Cells in Patients Exhibiting a Poor Response to Erythropoietin Therapy

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**Abstract.** Resistance to recombinant human erythropoietin occurs in a small but important proportion of hemodialysis patients. This may be due to increased immune activation because pro-inflammatory cytokines inhibit erythropoiesis *in vitro*. Using FACScan flow cytometry, the proportion of PMA/ionomycin-stimulated T cells expressing cytokines *ex vivo* was compared in 18 poor responders to erythropoietin, 14 good responders to erythropoietin, and 14 normal controls. CD4<sup>+</sup> T cells from poor responders expressed more interferon- $\gamma$  (IFN- $\gamma$ ;  $19 \pm 6\%$ ) compared with good responders ( $11 \pm 6\%$ ,  $P < 0.001$ ) and controls ( $12 \pm 6\%$ ,  $P < 0.01$ ). Similarly, CD4<sup>+</sup> T cells from poor responders expressed more tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; poor responders:  $51 \pm 19\%$  versus good responders:  $27 \pm 15\%$  [ $P < 0.01$ ] and controls:  $30 \pm 19\%$  [ $P < 0.01$ ]). CD4<sup>+</sup> expression of IL-10 was also enhanced (poor responders:  $1.6 \pm 1.1\%$  versus good responders:  $0.7 \pm 0.6\%$  [ $P < 0.05$ ] and controls:  $0.5 \pm 0.2\%$  [ $P < 0.01$ ]).

Likewise, CD4<sup>+</sup> expression of interleukin-13 (IL-13) was increased (poor responders:  $4.4 \pm 4.2\%$  versus good responders:  $1.6 \pm 1.7\%$  [ $P < 0.05$ ] and controls:  $1.6 \pm 1.5\%$  [ $P < 0.05$ ]). CD8<sup>+</sup> T cells from poor responders also showed enhanced expression of cytokines. For IFN- $\gamma$ , poor responder expression was  $48 \pm 20\%$  compared with  $31 \pm 17\%$  ( $P < 0.05$ ) for good responders and  $23 \pm 15\%$  ( $P < 0.01$ ) for controls. TNF- $\alpha$  expression for poor responders was  $41 \pm 21\%$  versus  $25 \pm 14\%$  for good responders ( $P < 0.05$ ) and  $21 \pm 15\%$  for controls ( $P < 0.01$ ). IL-10 expression for poor responders was  $2.0 \pm 1.2\%$  (good responders:  $0.7 \pm 0.6\%$  [ $P < 0.01$ ]; controls:  $0.5 \pm 0.2\%$  [ $P < 0.001$ ]). These data indicate that T cells from poor responders are in an enhanced activation state possibly as a result of chronic inflammation. In the absence of any other cause (such as iron deficiency), the overproduction of cytokines may account for hyporesponsiveness to erythropoietic therapy in patients with renal failure.

Anemia is a major complication of end-stage renal disease (ESRD), occurring primarily as a result of reduced erythropoietin production from the peritubular cells of the kidney (1). Recombinant human erythropoietin (rh-Epo) therapy has successfully corrected the anemia in many ESRD patients. The desired target hemoglobin (Hb) level is usually achieved with relatively low doses (50 to 150 IU/kg per wk) of rh-Epo. However, up to 25% of dialysis patients are relatively resistant to therapy (2) and require higher doses (>200 IU/kg per wk) to reach the target Hb level. Furthermore, 5 to 10% of patients do not reach the appropriate Hb level, even on high doses of rh-Epo (3).

Failure to achieve the target Hb has major clinical consequences because Hb levels predict morbidity and mortality in

the dialysis population (4). Anemia also affects cognitive function, quality of life, exercise capacity, and cardiac function in patients with ESRD (5). The main factors that affect the patients' response to rh-Epo include iron deficiency, insufficient rh-Epo dose, underdialysis, and comorbid factors such as acute infection (3). Having corrected for these, a significant number of dialysis patients remain hyporesponsive to rh-Epo. It has been postulated that the marked variability to rh-Epo therapy is caused by enhanced immune activation and the production of pro-inflammatory cytokines (6,7). In support of this hypothesis, the pro-inflammatory cytokines, interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-1 (IL-1), have been shown to suppress erythropoiesis *in vitro* (8–10). However, to date, evidence of enhanced production of these specific cytokines from HD patients that respond poorly to rh-Epo has not been demonstrated.

This study examines the activation state of T lymphocytes from patients failing to respond to rh-Epo. Specifically, the expression of Th1 and Th2 cytokines at the single cell level was assessed in three distinct groups, namely (1) uremic poor responders to rh-Epo, (2) uremic good responders to rh-Epo, to exclude the potential influence of uremia *per se*, and (3) healthy control subjects.

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## Materials and Methods

### Subjects

Thirty-two hemodialysis (HD) patients and 14 normal controls were included in this study. Two distinct groups of HD patients were recruited: (1) 14 HD patients responding well to rh-Epo with Hb > 10 g/dl and rh-Epo dose < 150 IU/kg per wk and (2) 18 HD patients responding poorly to rh-Epo with Hb < 10 g/dl and rh-Epo dose > 300 IU/kg per wk. The patients were all maintained on the same dose of subcutaneous rh-Epo for at least 3 mo before investigation. The US guidelines define hyporesponsiveness as a failure to achieve the target Hb level at a rh-Epo dose of 300 IU/kg per wk when administered subcutaneously and 450 IU/kg per wk when administered intravenously (11). Thus, our selection criteria for the poor responder group fulfilled this definition. The study was approved by the Research Ethics Committee of King's College Hospital, and informed consent was obtained from all subjects.

The causes of renal failure in the patient population were as follows: hypertensive nephropathy ( $n = 6$ ), diabetes type II ( $n = 12$ ), chronic glomerulonephritis ( $n = 3$ ), ischemic nephropathy ( $n = 1$ ), polycystic kidney disease ( $n = 1$ ), renal amyloid ( $n = 1$ ), renovascular disease ( $n = 2$ ), and unknown ( $n = 6$ ). Of the 14 good responders, 4 were taking ramipril, 1 was taking quinapril, 1 was taking enalapril, 1 was taking perindopril, and 2 were taking irbesartan. For the 18 poor responders, 7 were taking ramipril, 5 were taking irbesartan, 1 was taking enalapril, and 2 were taking perindopril. Patients with autoimmune disease, malignancy, hematologic disorders (such as myelodysplastic syndrome and myeloma), systemic vasculitis, iron deficiency, overt acute or chronic infection (such as sepsis related to vascular access, abscess, etc), or those taking immunosuppressive therapy were all excluded from the study. All patients were given intravenous iron supplementation as required, to maintain their serum ferritin levels above 200  $\mu\text{g/L}$ , (as per the protocol in our unit). Serum  $\text{B}_{12}$  and folate levels were measured on all patients in the study and were in the normal range. The patients were dialyzed three times per week for 3 to 5 h, and all used a cellulose diacetate dialyzer. The three groups were age-matched and gender-matched as far as possible.

### Cell Preparation and Culture Conditions

Blood from HD patients was taken immediately before a dialysis session. Peripheral blood mononuclear cells (PBMC) were isolated by lymphoprep (Robbins Scientific, W Midlands, UK) density gradient centrifugation at  $800 \times g$  for 20 min at  $18^\circ\text{C}$ . Cells were washed in Hanks balanced salt solution (HBSS; Life Technologies BRL, Life Technologies Ltd, Paisley, UK) ( $600 \times g$ , 10 min,  $4^\circ\text{C}$ ). Two additional washes with HBSS were performed ( $200 \times g$ , 10 min,  $4^\circ\text{C}$ ). PBMC were cultured at  $2 \times 10^6/\text{ml}$  in RPMI-1640 medium (Life Technologies Ltd, Paisley, UK) supplemented with 1% sodium pyruvate (Sigma Chemical Co., Poole, Dorset, UK), 1% minimal nonessential amino acids (Sigma Chemical Co., Poole, Dorset, UK), 0.5% gentamicin (Life Technologies BRL, Life Technologies Ltd, Paisley, UK), and 10% human AB serum (Serotec, Oxford, UK). The cultures were set up either in media alone (unstimulated samples) or in the presence of ionomycin (400 ng/ml; Sigma Chemical Co., Poole, Dorset, UK) plus PMA (10 ng/ml; Sigma Chemical Co., Poole, Dorset, UK) (stimulated samples). The protein transport inhibitor, brefeldin A (5  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co., Poole, Dorset, UK) was included in all cultures.

### Cytokine Measurement by Flow Cytometry

**T Cell Cytokine Expression of PBMC from HD Patients and Controls.** T cell cytokine expression was determined using the flow cytometric method developed by Jung *et al.* (12) and Prussin *et al.* (13). After 16-h culture ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ), the PBMC were washed twice using phosphate-buffered saline (PBS; Life Technologies Ltd, Paisley, UK) containing 0.1% bovine serum albumin (BSA; Sigma Chemical Co., Poole, Dorset, UK;  $200 \times g$ , 5 min,  $4^\circ\text{C}$ ). As cell surface marker expression was to be correlated with cytokine generation, cells were initially stained with mouse anti-human PerCP-conjugated CD3 (Becton Dickinson (UK) Ltd, Cowley, Oxford, UK), together with either mouse anti-human PE-conjugated CD8 or mouse anti-human FITC-conjugated CD8 (both Becton Dickinson (UK) Ltd, Cowley, Oxford, UK). In addition, control cell samples were labeled with the appropriate FITC-, PE-, and PerCP-labeled isotype-matched antibodies. The cells were incubated with the conjugated antibodies for 30 min at  $4^\circ\text{C}$  in the dark at room temperature (RT), washed with PBS containing 0.1% BSA, and fixed with 4% formaldehyde solution for 20 min at RT in the dark. Cells were washed to remove the fixative and then permeabilized using 0.5% saponin (Sigma Chemical Co., Poole, Dorset, UK) in PBS containing 1% BSA for 10 min at RT in the dark. After permeabilization, cells were washed ( $200 \times g$ , 5 min,  $4^\circ\text{C}$ ), then stained for intracellular cytokines using the following monoclonal antibodies: PE-labeled TNF- $\alpha$ , IL-4, IL-10, or IL-13, or FITC-labeled IFN- $\gamma$  (Becton Dickinson (UK) Ltd, Cowley, Oxford, UK; 30 min, RT, in the dark). Cells were washed with 0.5% saponin in PBS containing 1% BSA and resuspended in 1% paraformaldehyde (Sigma Chemical Co., Poole, Dorset, UK), ready for flow cytometric analysis.

### Flow Cytometry

The labeled PBMC samples were analyzed on the same day as staining. Measurements were performed on a Becton Dickinson FAC-Scan flow cytometer using Cellquest software 3.1. Initial gating was performed using forward and side scatter to identify T lymphocytes. For each specimen, a total of 20,000 events in the CD3 gate were collected. The percentage of CD8 cells positive for a cytokine was calculated by dividing the number of events in the CD8 positive/cytokine positive quadrant by the sum of events in both the CD8-positive/cytokine-positive quadrant and the CD8-positive/cytokine-negative quadrant. PMA/ionomycin activation leads to downregulation of the CD4 surface antigen. Therefore, CD4-positive cells were identified by gating on the CD3-positive cell population and excluding the CD8-positive cells, thereby assuming that  $\text{CD3}^+/\text{CD8}^-$  cells were  $\text{CD4}^+$  T cells. The percentage of CD4 cells that were positive for a cytokine was determined as described for CD8.

### Cytokine Measurement by Flow Cytometry:

#### Reproducibility Studies

The reproducibility of the flow cytometry cytokine assay was assessed by analyzing five samples processed on the same day from one subject. Cells were stimulated with PMA/ionomycin, and cell staining was carried out as described. The cells were stained for IFN- $\gamma$  and TNF- $\alpha$ . In addition, five samples were processed (using the methodology described) on five consecutive days from the same subject. The cells were stained for IFN- $\gamma$ .

### Statistical Analyses

Statistical analyses were carried out using Prism V3.0 statistical software (Graphpad, San Diego, CA). Results are expressed as mean  $\pm$  SD. Differences between the three subject groups were first ana-

lyzed using one-way ANOVA statistical test followed by the Bonferroni post-test to compare all pairs of group means. For analysis of two groups, the *t* test for unpaired data were used (after checking for normality using the Kolmogorov-Smirnov test). Results were considered significant at  $P < 0.05$ .

## Results

### *Hemodialysis and Clinical Comparisons between Good and Poor Responders to rh-Epo*

The mean time on HD did not vary between the two groups ( $49 \pm 37$  mo for good responders *versus*  $54 \pm 39$  mo for poor responders;  $P = 0.73$ ). The urea reduction ratio was not significantly different between the good and poor responder groups ( $69 \pm 12\%$  and  $65 \pm 7\%$ , respectively;  $P = 0.19$ ). Serum ferritin levels were similar between the two groups ( $545 \pm 334$   $\mu\text{g/L}$  for good responders *versus*  $567 \pm 303$   $\mu\text{g/L}$  for poor responders;  $P = 0.84$ ). Likewise there was no significant difference in PTH levels between the two groups ( $337 \pm 342$  pg/ml for good responders *versus*  $368 \pm 190$  pg/ml for poor responders;  $P = 0.76$ ), nor was there any difference in serum albumin levels ( $39 \pm 3$  g/L for good responders *versus*  $36 \pm 6$  g/L for poor responders;  $P = 0.13$ ).

### *Demographic and Hematologic Characteristics of the Three Subject Groups*

The demographic and hematologic characteristics of the three groups studied are shown in Table 1. The three subject groups were age-matched as far as possible, and there was no statistical difference between the mean ages of the three groups. The mean rh-EPO dose in the poor responders was significantly higher than the mean of the HD patients responding well to rh-EPO. The poor responders were significantly more anemic than the good responders; hence, the rh-EPO/Hb ratio was significantly different between the two HD groups

(Table 1). The selection process for the two HD groups had therefore generated two distinct HD populations in terms of dose and response to rh-EPO with no overlap.

C-reactive protein (CRP) levels were found to be higher in the poor responders compared with both the good responders and normal controls (Table 1). The white cell count in the three subject groups did not differ significantly (Table 1). However, lymphocyte numbers were significantly lower in both the poor responders and the good responders compared with the normal controls. There was no significant difference in the lymphocyte count between good and poor responders.

### *Reproducibility Studies on the T Cell Cytokine Expression Assay*

The results of the within-subject reproducibility study are shown in Table 2. For five samples from the same patient assayed on the same day, the coefficient of variance was below 15% for the percentage of IFN- $\gamma$  and TNF- $\alpha$  expressed. For five samples from the same patient assayed on separate days, the coefficient of variance was below 7% for the percentage of IFN- $\gamma$  expressed.

### *T Cell Activation Demonstrated at the Single Cell Level in the Three Subject Groups*

*Ex vivo* T cell expression of cytokines was studied to compare immune reactivity among the three subject groups. T cell cytokine expression was determined on unstimulated and ionomycin/PMA-stimulated mixed leukocyte cultures.

### *Poor Responders to rh-Epo Express More TNF- $\alpha$ from Unstimulated CD8<sup>+</sup> T Cells and IL-10 from Unstimulated CD4<sup>+</sup> T Cells*

The results of cytokine expression in unstimulated mixed leukocyte cultures are shown in Table 3. CD8<sup>+</sup> T cells from

Table 1. Demographic and hematologic characteristics of subjects (mean  $\pm$  SD)<sup>a</sup>

|   | Normal Controls<br>(n = 14) | Good Responders<br>(n = 14) | Poor Responders<br>(n = 18) | One-way ANOVA<br>Variance Ratio (F),<br>Probability (P) |
|---|-----------------------------|-----------------------------|-----------------------------|---|
| Age (yr)                                    | 54 $\pm$ 22                 | 60 $\pm$ 15                 | 59 $\pm$ 13                 | F = 0.585<br>P = 0.5613                                 |
| Hb (g/dl)                                   | 13.7 $\pm$ 1.0              | 12.8 $\pm$ 1.2              | 9.0 $\pm$ 1.0 <sup>ec</sup> | F = 90.6<br>P < 0.0001                                  |
| CRP (mg/L)                                  | 3 $\pm$ 1                   | 6 $\pm$ 5                   | 28 $\pm$ 23 <sup>ec</sup>   | F = 13.5<br>P < 0.0001                                  |
| Rh-Epo dose (U/kg per wk)                   | N/A                         | 118 $\pm$ 35                | 377 $\pm$ 107 <sup>c</sup>  | N/A   |
| Rh-Epo dose/Hb ratio                        | N/A                         | 9 $\pm$ 3                   | 49 $\pm$ 18 <sup>c</sup>    | N/A   |
| White cell count ( $\times 10^9/\text{L}$ ) | 6.5 $\pm$ 1.7               | 5.9 $\pm$ 1.4               | 6.5 $\pm$ 1.9               | F = 0.5058<br>P = 0.6066                                |
| Lymphocytes ( $\times 10^9/\text{L}$ )      | 2.1 $\pm$ 0.3               | 1.2 $\pm$ 0.4 <sup>f</sup>  | 1.2 $\pm$ 0.5 <sup>e</sup>  | F = 22.4<br>P < 0.0001                                  |

<sup>a</sup> Samples were taken for clinical and hematologic analysis at the same time as cytokine and immunofluorescence analysis. Hb, hemoglobin; CRP, C-reactive protein; rh-Epo, recombinant human erythropoietin; N/A, not applicable.

<sup>b</sup>  $P < 0.01$  and <sup>c</sup>  $P < 0.001$  for poor responders *versus* good responders.

<sup>d</sup>  $P < 0.01$  and <sup>e</sup>  $P < 0.001$  for poor responders *versus* normal controls.

<sup>f</sup>  $P < 0.001$  for good responders *versus* normal controls.

Table 2. Intrasubject reproducibility studies on cytokine expression<sup>a</sup>

|                            | %    | %    | %    | %    | %    | Mean (%) | Standard Deviation | Coefficient of Variance (%) |
|----------------------------|------|------|------|------|------|----------|--------------------|-----------------------------|
| Same day <sup>b</sup>      |      |      |      |      |      |          |                    |                             |
| CD8/IFN- $\gamma$          | 15.1 | 14.7 | 13.6 | 14.6 | 14.9 | 14.58    | 0.58               | 4.0                         |
| CD4/IFN- $\gamma$          | 11.0 | 11.2 | 14.3 | 14.2 | 15.3 | 13.20    | 1.97               | 14.9                        |
| CD8/TNF- $\alpha$          | 19.6 | 18.9 | 19.2 | 20.1 | 20.8 | 19.72    | 0.75               | 5.3                         |
| CD4/TNF- $\alpha$          | 31.7 | 31.9 | 28.1 | 31.2 | 29.6 | 30.50    | 1.62               | 3.8                         |
| Separate days <sup>c</sup> |      |      |      |      |      |          |                    |                             |
| CD8/IFN- $\gamma$          | 12.6 | 13.3 | 13.2 | 11.8 | 10.9 | 12.36    | 1.01               | 5.3                         |
| CD4/IFN- $\gamma$          | 11.4 | 10.8 | 12.2 | 13.1 | 11.9 | 11.88    | 0.86               | 7.3                         |

<sup>a</sup> Reproducibility results of the flow cytometry cytokine assay using PMA/ionomycin-stimulated cells.

<sup>b</sup> Five measurements determined on the same day using the same subject.

<sup>c</sup> Measurements on five separate days on the same subject.

Table 3. Spontaneous cytokine expression in subjects (mean  $\pm$  SD)<sup>a</sup>

| T Cell Subset/cytokine Expression | Normal Controls (n = 14) (%) | Good Responders (n = 14) (%) | Poor Responders (n = 18) (%) | One-Way ANOVA Variance ratio (F), Probability (P) |
|-----------------------------------|------------------------------|------------------------------|------------------------------|---|
| CD8/IFN- $\gamma$                 | 0.3 $\pm$ 0.3                | 0.2 $\pm$ 0.3                | 0.6 $\pm$ 0.8                | F = 2.661<br>P = 0.0820                           |
| CD4/IFN- $\gamma$                 | 0.1 $\pm$ 0.2                | 0.3 $\pm$ 0.7                | 0.2 $\pm$ 0.3                | F = 0.717<br>P = 0.4943                           |
| CD8/TNF- $\alpha$                 | 0.3 $\pm$ 0.3                | 0.4 $\pm$ 0.4                | 1.5 $\pm$ 1.2 <sup>ce</sup>  | F = 9.179<br>P = 0.0005                           |
| CD4/TNF- $\alpha$                 | 0.7 $\pm$ 1.1                | 0.4 $\pm$ 0.5                | 0.7 $\pm$ 0.6                | F = 0.651<br>P = 0.5269                           |
| CD8/IL-4                          | 0.4 $\pm$ 0.4                | 0.4 $\pm$ 0.4                | 0.5 $\pm$ 0.8                | F = 0.298<br>P = 0.7439                           |
| CD4/IL-4                          | 0.3 $\pm$ 0.5                | 0.2 $\pm$ 0.2                | 0.3 $\pm$ 0.3                | F = 0.533<br>P = 0.6389                           |
| CD8/IL-10                         | 0.4 $\pm$ 0.4                | 0.9 $\pm$ 1.8                | 2.4 $\pm$ 3.0 <sup>d</sup>   | F = 3.700<br>P = 0.0333                           |
| CD4/IL-10                         | 0.1 $\pm$ 0.1                | 0.2 $\pm$ 0.3                | 0.5 $\pm$ 0.2 <sup>be</sup>  | F = 7.262<br>P = 0.0020                           |
| CD8/IL-13                         | 0.2 $\pm$ 0.4                | 0.1 $\pm$ 0.2                | 0.7 $\pm$ 1.1                | F = 3.293<br>P = 0.0472                           |
| CD4/IL-13                         | 0.1 $\pm$ 0.3                | 0.1 $\pm$ 0.1                | 0.1 $\pm$ 0.2                | F = 1.429<br>P = 0.2512                           |

<sup>a</sup> PBMC were taken prior to dialysis session and cultured for 18 h before cytokine determination by flow cytometry.

<sup>b</sup>  $P < 0.05$  and <sup>c</sup>  $P < 0.01$  for poor responders *versus* good responders.

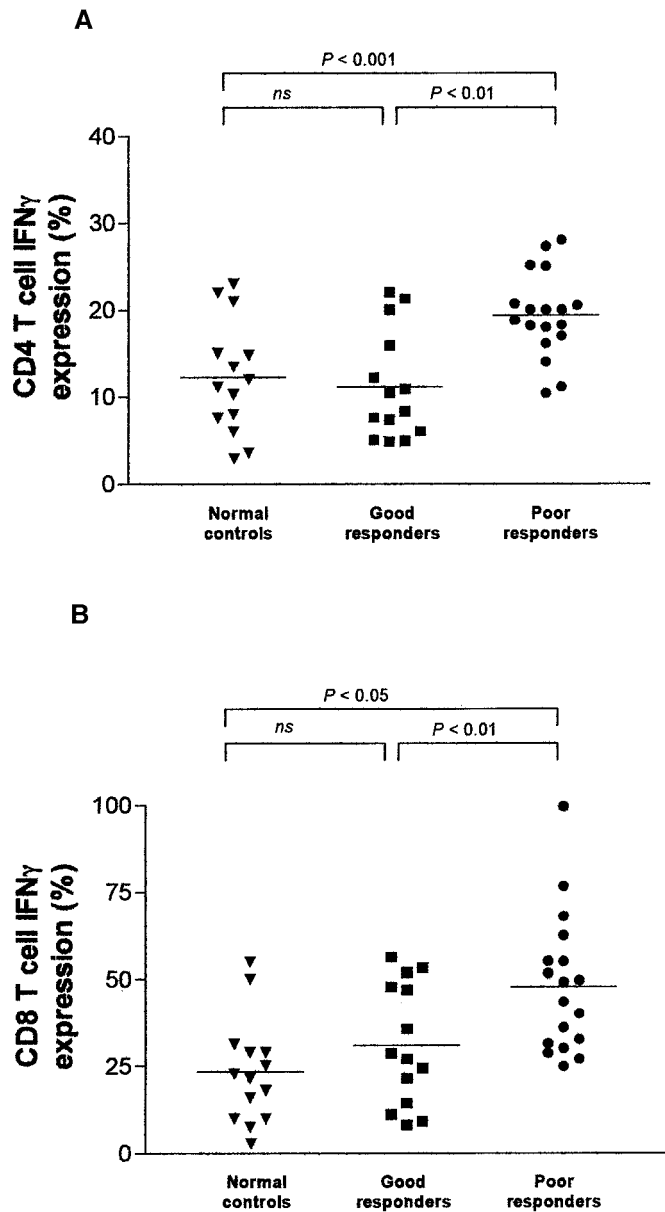
<sup>d</sup>  $P < 0.05$  and <sup>e</sup>  $P < 0.01$  for poor responders *versus* normal controls.

poor responders spontaneously expressed significantly more TNF- $\alpha$  compared with the other subject groups. In contrast, CD4<sup>+</sup> T cells from poor responders did not express significantly more TNF- $\alpha$  compared with good responders and normal controls. CD4<sup>+</sup> T cells from poor responders expressed significantly more IL-10 compared with other subject groups. For CD8<sup>+</sup> T cells, poor responders only expressed more IL-10 compared with the normal controls. Spontaneous expression of IL-4, IL-13, and IFN- $\gamma$  was similar in the three subject groups.

#### Poor Responders to rh-Epo Express more IFN- $\gamma$ from Both Stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T Cells

CD4<sup>+</sup> T cells from poor responders to rh-Epo were found to express significantly more IFN- $\gamma$  (19  $\pm$  6%) compared with both good responders (11  $\pm$  6%,  $P < 0.001$ ) and normal controls (12  $\pm$  6%,  $P < 0.01$ ) (Figure 1A (one-way ANOVA: F = 9.22, degrees of freedom = 2,  $P = 0.0004$ )). There was no difference between the good responders and normal controls. Significantly more CD8<sup>+</sup> T cells from poor responders ex-





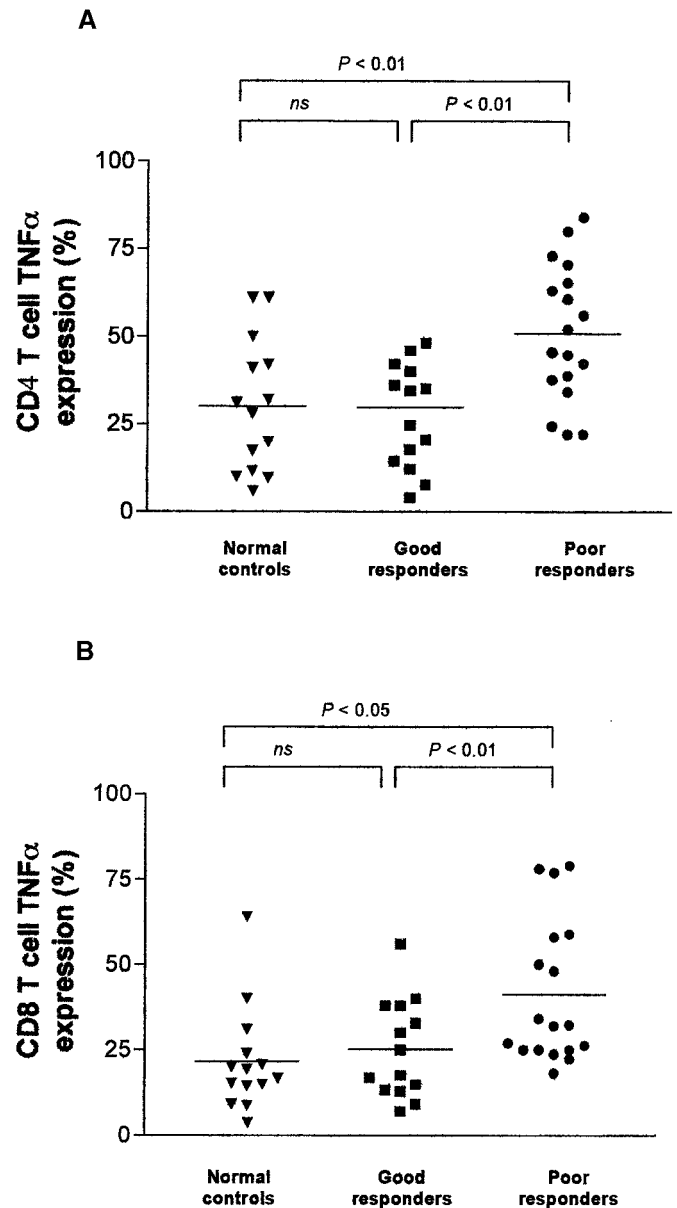
**Figure 1.** (A) Percentage of CD4<sup>+</sup> T cells in normal controls, good responders, and poor responders to erythropoietin that express interferon- $\gamma$  (IFN- $\gamma$ ). (B) Percentage of CD8<sup>+</sup> T cells in normal controls, good responders, and poor responders to erythropoietin that express IFN- $\gamma$ . Horizontal bars indicate the mean percentage values.

pressed IFN- $\gamma$  ( $41 \pm 21\%$ ) compared with good responders ( $31 \pm 17\%$ ,  $P < 0.05$ ) and normal controls ( $23 \pm 15\%$ ,  $P < 0.01$ ) (Figure 1B (one-way ANOVA:  $F = 7.98$ , degrees of freedom = 2,  $P = 0.0011$ )).

#### Enhanced TNF- $\alpha$ Expression from Both Stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T Cells from Poor Responders to rh-Epo

The proportion of CD4<sup>+</sup> T cells from poor responders expressing TNF- $\alpha$  ( $51 \pm 19\%$ ) was significantly higher compared with good responders ( $27 \pm 15\%$ ,  $P < 0.01$ ) and normal

controls ( $30 \pm 19\%$ ,  $P < 0.01$ ) (Figure 2A; one-way ANOVA:  $F = 8.49$ , degrees of freedom = 2,  $P = 0.0008$ ). Likewise this pattern was also observed with CD8<sup>+</sup> T cells from poor responders (TNF- $\alpha$  expression frequency: poor responders:  $41 \pm 21\%$  versus good responders:  $25 \pm 14\%$  [ $P < 0.05$ ] and normal controls  $21 \pm 15\%$  [ $P < 0.01$ ]) (Figure 2B) (one-way ANOVA:  $F = 5.81$ , degrees of freedom = 2,  $P = 0.0059$ ). T cells from good responders to rh-Epo and normal controls exhibited a similar degree of TNF- $\alpha$  expression for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.



**Figure 2.** (A) Percentage of CD4<sup>+</sup> T cells in normal controls, good responders, and poor responders to recombinant human erythropoietin that express tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). (B) Percentage of CD8<sup>+</sup> T cells in normal controls, good responders, and poor responders to recombinant human erythropoietin that express TNF- $\alpha$ . Horizontal bars indicate the mean percentage values.

**Comparable IL-4 Expression in All Three Subject Groups**

IL-4 expression was similar in all three subject groups for both CD4<sup>+</sup> T cells (Figure 3A) (one-way ANOVA:  $F = 1.67$ , degrees of freedom = 2,  $P = 0.200$ ). For CD8<sup>+</sup> T cells, there was no statistical difference in IL-4 expression in the three subject groups according to the Bonferroni post test (Figure 3B) (one-way ANOVA:  $F = 3.32$ , degrees of freedom = 2,  $P = 0.046$ ).

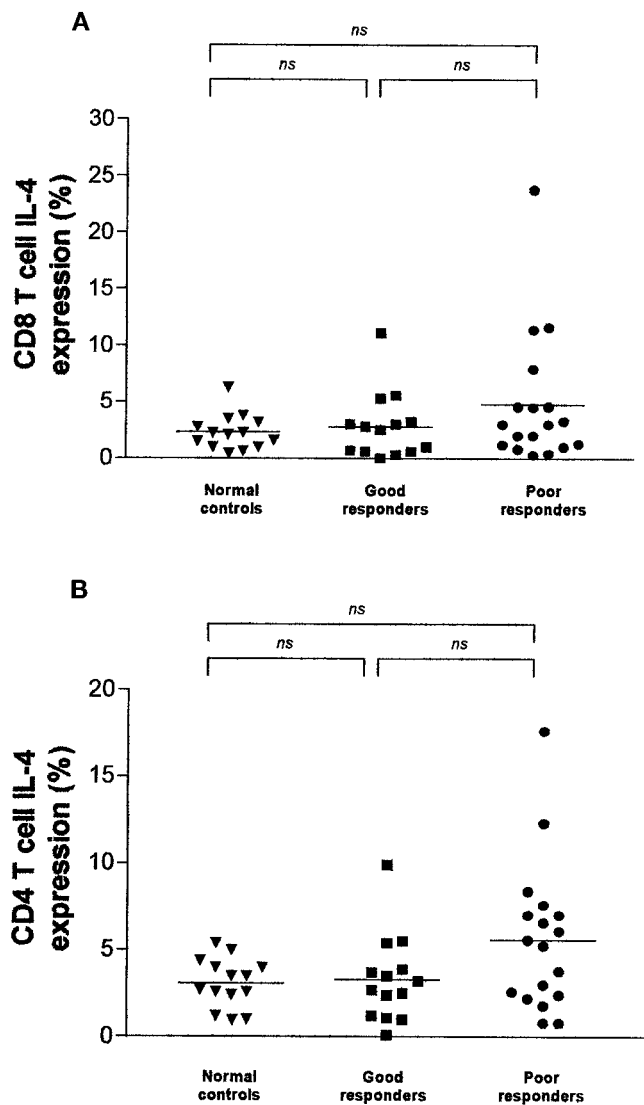
**Stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells from Poor Responders to rh-Epo Show Increased Expression of IL-10**

The intracellular detection of IL-10 showed that CD4<sup>+</sup> T cells from poor responders had increased numbers of cells expressing this cytokine ( $1.6 \pm 1.1\%$ ) compared with good responders ( $0.7$

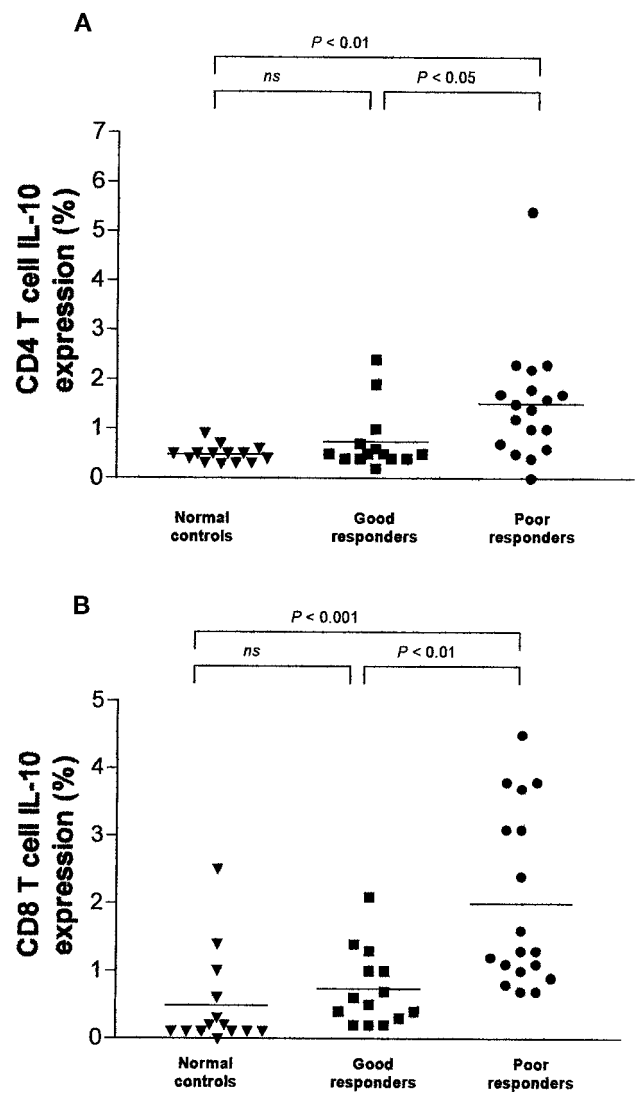
$\pm 0.6\%$ ,  $P < 0.05$ ) and normal controls ( $0.5 \pm 0.2\%$ ,  $P < 0.01$ ) (Figure 4A) (one-way ANOVA:  $F = 6.98$ , degrees of freedom = 2,  $P = 0.0024$ ). There was no difference in the frequency of expression between good responders and normal controls. For CD8<sup>+</sup> T cells, poor responders had a higher proportion of cells expressing IL-10 ( $2.0 \pm 1.2\%$ ) compared with both good responders ( $0.7 \pm 0.6\%$ ,  $P < 0.01$ ) and normal controls ( $0.5 \pm 0.2\%$ ,  $P < 0.001$ ) (Figure 4B) (one-way ANOVA:  $F = 11.92$ , degrees of freedom = 2,  $P < 0.0001$ ).

**Stimulated CD4<sup>+</sup> T Cells from Poor Responders to rh-Epo Show Increased Capacity to Express IL-13**

CD4<sup>+</sup> T cell expression of IL-13 was increased in poor responders compared with the other groups (poor responders:



**Figure 3.** (A) Percentage of CD4<sup>+</sup> T cells in normal controls, good responders, and poor responders to recombinant human erythropoietin that express interleukin-4 (IL-4). (B) Percentage of CD8<sup>+</sup> T cells in normal controls, good responders, and poor responders to recombinant human erythropoietin that express IL-4. Horizontal bars indicate the mean percentage values.



**Figure 4.** (A) Percentage of CD4<sup>+</sup> T cells in normal controls, good responders, and poor responders to recombinant human erythropoietin that express IL-10. (B) Percentage of CD8<sup>+</sup> T cells in normal controls, good responders, and poor responders to recombinant human erythropoietin that express IL-10. Horizontal bars indicate the mean percentage values.

4.4 ± 4.2% versus good responders: 1.6 ± 1.7% [*P* < 0.05] and normal controls: 1.6 ± 1.5% [*P* < 0.05]) (Figure 5A) (one-way ANOVA: *F* = 4.80, degrees of freedom = 2, *P* = 0.0131). CD8<sup>+</sup> T cells from poor responders had statistically higher expression of IL-13 compared with only the good responders (3.8 ± 3.1% versus 0.8 ± 0.7%, *P* < 0.01) (Figure 5B) (one-way ANOVA: *F* = 6.27, degrees of freedom = 2, *P* = 0.0041).

**Discussion**

This study has demonstrated for the first time that a poor response to rh-Epo is associated with enhanced T cell capacity to express IFN-γ, TNF-α, IL-10, and IL-13. The standard method for determining cytokine production is the detection of

secreted cytokines in culture supernatant by the enzyme-linked immunosorbent assay (ELISA). Using the ELISA methodology, we previously found that PBMC from poor responders secreted more IL-10 compared with good responders and normal control (14). However, no differences were found with the other cytokines, and this may have been due to reduced relative numbers of lymphocytes in the mixed leukocytes cultures because the uremic patients were lymphopenic. The advantage of cytokine determination by flow cytometry is that the method is independent of variations in the proportions of T cells to other mononuclear cells in mixed leukocyte cultures. Hence cytokine expression is determined on a single cell basis and expression can be assigned to either the CD4 or the CD8 T cell subset. A study on monocyte cytokine secretion also found that the flow cytometry method had a greater capacity for discrimination between subject groups compared with the ELISA method (15).

These results are particularly exciting because *in vitro* hematopoietic stem cell experiments have already established that IFN-γ (16) and TNF-α (17) inhibit erythroid cell development. Specifically, these cytokines inhibit the formation of erythroid colony forming units (CFU-E), the early developmental precursors of red cells that proliferate in the bone marrow. The *ex vivo* data described in this study support the concept that T cell-derived pro-inflammatory cytokines contribute to the development of rh-Epo-resistant anemia. Figure 6 depicts the hypothesis that we propose may account for rh-Epo hyporesponsiveness in terms of T cell activity. IFN-γ is the primary mediator of erythroid suppression, because it antagonizes the anti-apoptotic effect of Epo on CFU-E (10). Because IFN-γ is only produced by T cells, it is likely that activated T cells are pivotal in mediating suppression of erythropoiesis in poor responders to rh-Epo. The inhibitory action of TNF-α on CFU-E is thought to be indirect, mediated via the action of IFN-β (17). Although IL-10 has an antiinflammatory action (18), this cytokine actually has an inhibitory effect on human

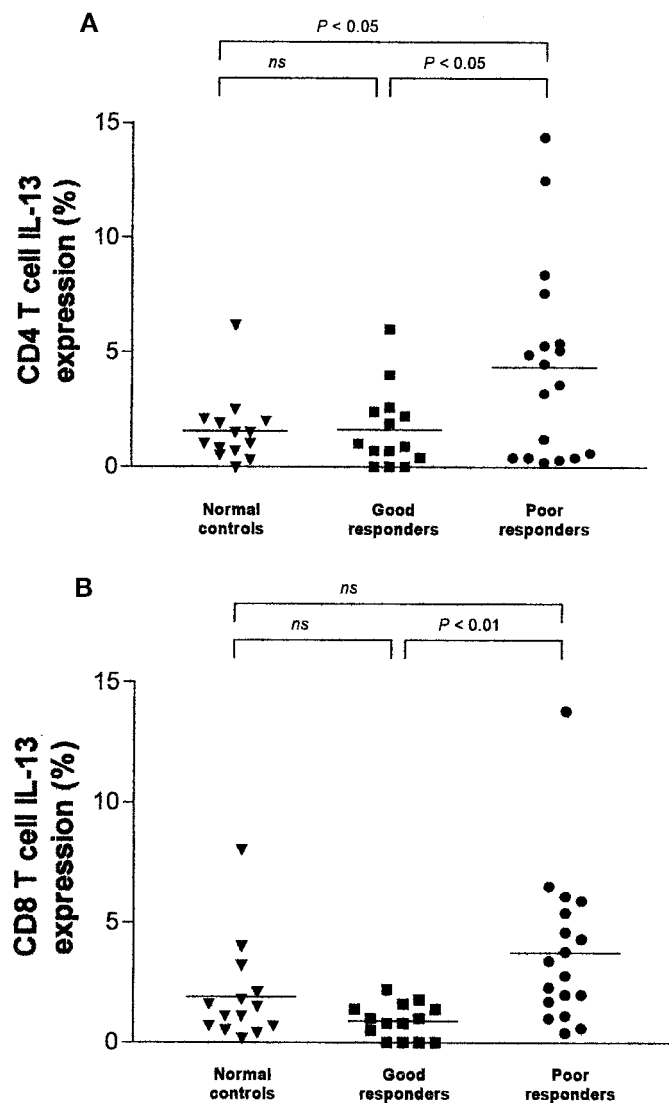


Figure 5. (A) Percentage of CD4<sup>+</sup> T cells in normal controls, good responders, and poor responders to recombinant human erythropoietin that express IL-13. (B) Percentage of CD8<sup>+</sup> T cells in normal controls, good responders, and poor responders to recombinant human erythropoietin that express IL-13. Horizontal bars indicate the mean percentage values.

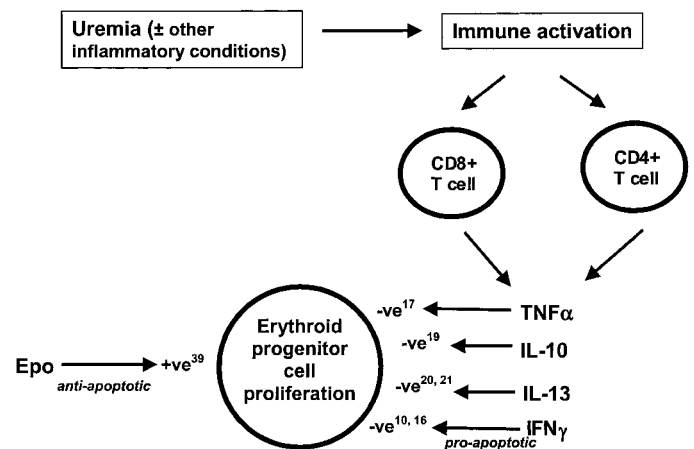


Figure 6. Proposed hypothesis of T cell cytokine-mediated inhibition of erythropoiesis in poor responders to recombinant human erythropoietin. -ve denotes inhibition of proliferation; +ve denotes stimulation of proliferation. Numbers refer to relevant references.

CFU-E formation (unpublished observation). Other studies have found that IL-10 inhibits the formation of erythroid burst-forming colony units, the erythroid cell progenitors that develop into CFU-E (19). IL-13 also inhibits the formation of CFU-E *in vitro* (20,21).

A number of factors have been implicated as potential causes of inflammation in the uremic patient. These include contaminated dialysate, bioincompatible membranes, impaired renal clearance of cytokines, unrecognized persistent infections, accumulation of advanced glycation end products, reduced plasma antioxidant activity, atherosclerosis *per se*, and co-morbid autoimmune diseases (22). With these in mind, the present study attempted to remove the potential bias of these factors on the results. Hence all HD patients who were recruited to the study used the same membrane, while the urea reduction ratio and time on HD was comparable in the two distinct HD groups. The bacterial count of the dialysate solution was also within British Renal Association guidelines. There was no clinical evidence of infection in recruited patients, patients with autoimmune diseases were excluded, and there was also no difference in the proportion of ACE inhibitor or angiotensin II blocker usage between the two groups. In addition, the iron status of the two HD groups was similar. Thus there was no obvious clinical cause of increased T cell reactivity in our poor responder cohort compared with the good responders. Nevertheless, the cytokine data indicate that the poor responder group appears to have nonspecific chronic inflammation, which is also reflected in the raised CRP levels in this group.

Analysis of cytokine production in HD patients has focused on changes in monocyte cytokine generation, such as IL-1 $\beta$  (23), TNF- $\alpha$ , IL-6 (15,24), IL-10 (15,24), and IL-12 (25). Increased production of monokines has been attributed to exposure of the blood to bioincompatible dialysis membranes (23) and the shear stress of dialysis (26). The microinflammatory environment in uremia may also lead to monocyte activation because predialysis patients show inflammatory activation (27). More recent studies on T cells from hemodialysis patients have also found changes in cytokine production. These changes have been related to the T helper cell functional dichotomy first described by Mosmann *et al.* (28). CD4<sup>+</sup> Th1 type cells are characterized by the production of high levels of IFN- $\gamma$ , which mediates pro-inflammatory reactions and helps B cells to produce IgG2a. CD4<sup>+</sup> Th2 cells secrete IL-4, which promotes B cell antibody production of IgG1 and IgE (29). The data in the literature are conflicting. Two studies have shown that T cells from HD patients secrete more IFN- $\gamma$  compared with normal controls (30,31). These results led the authors to conclude that T cell activation in HD patients follows the Th1 pattern (30,31). Another study found that T cell IFN- $\gamma$  secretion was reduced in HD patients, while their T cells secreted more IL-4 and IL-10 (IFN- $\gamma$  secretion was restored by neutralization of the IL-4 and IL-10) (32). This suggested that T cells are polarized toward the Th2 phenotype in HD patients (32). The present study did not find any difference in CD4<sup>+</sup> T cell secretion of IFN- $\gamma$  nor of IL-4 between the healthy controls and the HD patients who were classified as good responders

to rh-Epo. Poor responders to rh-Epo expressed significantly more IFN- $\gamma$  compared with the other two groups (Th1 cytokine). However, CD4<sup>+</sup> T cells from poor responders also secreted more IL-10 and IL-13, and these have been described as Th2-type cytokines; hence, the poor response cytokine secretion profile does not fit into the Th1/Th2 dichotomy.

Distinct subsets of CD8<sup>+</sup> T cells have also been described in rodents (33,34) and humans (35,36). Analogous to the Th1/Th2 terminology, these subsets have been defined as Tc1 (IFN- $\gamma$ , no IL-4), Tc0 (IFN- $\gamma$ , IL-4), and Tc2 (no IFN- $\gamma$ , IL-4). The CD8<sup>+</sup> T cell cytokine secretion pattern of poor responders appears to follow a type 1 cytokine profile, because poor responders show increased expression of IFN- $\gamma$ , with no increase in IL-4. However, CD8<sup>+</sup> cells from poor responders did express more IL-10 and IL-13 compared with good responders, indicating that elevated cytokine secretion from poor responders does not fit into a distinct CD8 T cell subset.

Elevated plasma levels of pro-inflammatory cytokines are predictive of mortality in dialysis patients (37). We have recently shown that a poor response to erythropoietin is associated with increased mortality (14). Furthermore, inflammation and malnutrition have been linked to increased atherosclerotic cardiovascular disease in ESRD (38). Minimizing the chronic inflammation observed in ESRD and would have obvious benefits on patient outcome, possibly as a result of improving anemia. Future strategies aimed at reducing T cell production of cytokines that inhibit erythropoiesis may have a major role in improving the response to rh-Epo.

In conclusion, this study has demonstrated that T cells from poor responders have an enhanced capacity to express IFN- $\gamma$ , TNF- $\alpha$ , IL-10, and IL-13 compared with good responders to rh-Epo. These four cytokines are known to have an anti-proliferative effect on erythroid progenitors *in vitro*. It is therefore proposed that increased production of these cytokines may account for rh-Epo hyporesponsiveness.

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