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T-cell activation follows Th1 rather than Th2 pattern in haemodialysis patients

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Abstract

Background. Patients on chronic intermittent haemodialysis (HD) show an impaired cellular and humoral immune response that clinically appears with frequent infectious complications and low vaccination responses. This immune defect strongly correlates with reduced *in vitro* proliferative responses of T cells. The defect is localized in antigen presenting cells, which show a decreased co-stimulatory activity. Furthermore, the impaired immune response correlates with an increased production of pro-inflammatory cytokines. In response to primary activation, CD4 positive T helper (Th) cells mainly differentiate into either Th1 or Th2 cells. Th1 cells support cell mediated immunity whereas Th2 cells enhance humoral immune responses. Since both types of responses mutually inhibit each other, the impaired humoral immune response seen in HD patients could either be due to a reduced number of Th2 cells or to a predominant Th1 response.

Methods. We analysed the Th cell profile in HD patients using flow cytometry. Monocytic cytokine expression was analysed using both flow cytometry and enzyme linked immunoadsorbant assays.

Results. Our data demonstrate that the cytokine differentiation profile in circulating T cells from HD patients is dysregulated and characterized by an increase in Th1 cells, but a normal amount of Th2 cells. Moreover, the skewed helper cell responses correlate with a higher percentage of monocytes capable of secreting the Th1 promoting cytokine interleukin 12 (IL-12).

Conclusions. Our findings contribute to a better understanding of the pathogenesis of impaired cellular immune functions in dialysis patients and, in particular, the decreased antibody production after vaccination. They provide a link between overproduction of pro-inflammatory cytokines (IL-12) and imbalanced T-cell activation.

Keywords: haemodialysis; Th1/Th2

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Introduction

End-stage renal failure induces a clinical state of immunodeficiency with a higher incidence of infections and a higher mortality due to infectious complications compared with the normal population [1]. This immunodeficiency is further characterized by an impaired response to vaccinations, such as against hepatitis B or tetanus [2,3]. The *in vivo* humoral immune response of patients correlates well with an altered activation of T cells. The defect was mainly found to reside in antigen presenting cells (APCs) [4], since *in vitro* it can be abrogated by the application of a co-stimulatory signal of the B7/CD28 pathway [5]. Apart from an altered co-stimulatory capacity of monocytes, we and others found an increased production of pro-inflammatory cytokines such as interleukin 6 (IL-6), tumour necrosis factor α (TNFα) and IL-1β in monocytes from patients undergoing chronic intermittent haemodialysis (HD) [6,7]. Both reduced co-stimulation and aberrant expression of cytokines are mainly induced by uraemia and further modified by regular contact of blood with artificial dialyser membranes, complement activation and recurrent exposure to pyrogens [6,8,9]. Therefore, these patients are thought to be in a chronic state of inflammation. Interestingly, similar to the decreased co-stimulatory capacity, the extent of cytokine expression also correlates with the severity of the humoral immunological disorder [7].

In response to antigens, naive CD4 T cells differentiate into effector T helper (Th) cells. Based on their pattern of cytokine production and their functional responses, Th cells can be subdivided into those that participate in cell-mediated immune responses such as delayed type of hypersensitivity (DTH) reactions and macrophage activation (Th1 subset), and those releasing cytokines that induce B cells to secrete antibodies (Th2 subset). Various autoimmune diseases are associated with an altered pattern of Th1 and Th2 cells in that systemic lupus erythematosus (SLE) and Wegener's granulomatosis (WG) are characterized by a shift in either Th2 or Th1 direction, respectively [10,11]. At present, several factors are known to polar-

ize the differentiation of Th cells into either Th1 or Th2 direction, these include the co-stimulatory action of APCs, the cytokine environment, altered peptide ligands and the antigen dose. It is clear that the APC-derived cytokine IL-12 directs differentiation to a Th1 phenotype, while IL-4 can drive differentiation to a Th2 phenotype [12,13].

Since both co-stimulation via B7 molecules and cytokine production are altered in HD patients and both are factors that determine the outcome of a Th cell response, it is intriguing to speculate whether these alterations might bias Th cell development in HD patients. Both types of Th cell responses mutually inhibit each other. Thus, in addition to the globally reduced T-cell activation, the impaired humoral immune response seen in HD patients could either be due to a reduced number of Th2 cells or to a predominant Th1 response. Our objective in the present study was to analyse the outcome of the Th1 or Th2 cell responses in HD patients compared with healthy controls and to place them in the context of the altered cellular characteristics and impaired immune status seen in HD patients.

Subjects and methods

Subjects

Twenty patients, 13 men and seven women, on chronic HD treatment (>3 months) for end-stage renal failure were studied. The average age was 56.5 ± 12.8 years. The control group consisted of 10 healthy individuals, among them four men and six women. The average age of the control group was 32.9 ± 7.3 years. The analysis of IL-12 production was restricted to 11 patients and six controls.

End-stage renal failure had occurred as a result of the following diseases: chronic glomerulonephritis ($n=4$), chronic pyelonephritis ($n=3$), diabetic nephropathy ($n=5$), polycystic kidney disease ($n=2$), malignant nephrosclerosis ($n=1$), focal segmental glomerulosclerosis ($n=1$), Alport's syndrome ($n=1$), chronic interstitial nephritis ($n=1$), unknown ($n=2$). Patients with systemic vasculitis, those showing evidence of intercurrent infection or malignancy, or those taking immunosuppressive medication were excluded.

Dialysis was performed using bicarbonate dialysate and GFS plus 20 dialyzers (Gambro, Hechingen, Germany) without reuse. Mean time on dialysis was 3.85 ± 0.37 h thrice weekly. The dialysis dose was calculated according to Daugirdas [14]. Mean Kt/V (double-pool) was 1.32 ± 0.33. All patients gave informed consent.

Cell preparation and culture conditions

Twenty millilitres of blood were collected into plastic syringes containing 1000 U of sodium heparin (Braun, Melsungen, Germany) before the start of HD. Blood samples were placed on ice immediately and peripheral blood mononuclear cells (PBMC) were prepared on a Ficoll density gradient (d = 1.077, Linaris, Bettingen, Germany) within 30 min. Cells were cultured at 2×10^6 cells/ml in RPMI 1640 (PAA, Cölbe, Germany) supplemented with 5% fetal calf serum (Biochrom, Berlin, Germany), 2 mM glutamine, and antibiotics (PAA). Culture media and all reagents were tested for absence of endotoxins. PBMC were polyclonally stimulated for 4 h using 20 ng/ml phorbolmyristate acetate (PMA, Sigma, Deisenhofen, Germany) and 1 μM ionomycin (Sigma) in the presence of the intracellular transport inhibitor monensin (2 μM, Sigma). Alternatively cells were propagated for 1 week after an initial stimulus by 62.5 ng/ml phytohaemagglutinin-L (PHA-L, Biochrom, Berlin, Germany). After 24 h of culture the supernatants were harvested for determination of IL-2 and IFNγ secretion and were replaced by supplemented medium containing 100 U/ml of recombinant IL-2 (Strathmann Biotech, Hannover, Germany). After a medium change at day 3 cells were supplied with medium lacking IL-2 from day 5 to day 8. On day 8, IL-4 and interferon γ (IFNγ) producing T cells were identified after restimulation of cells with PMA/ionomycin in the presence of 2 μM monensin as described above.

For assessment of IL-12, PBMC were incubated for 2 h with 1000 U/ml of IFNγ (Strathmann Biotech) followed by 10 h with 1 μg/ml lipopolysaccharide (LPS, *Escherichia coli*, Sigma). For flow cytometry, 2 μM monensin was added together with LPS.

When monocytes were stimulated for flow cytometry, PBMC were cultured in plastic dishes coated with poly-2-hydroxyethyl-methacrylate (PHEMA, Sigma) to prevent adherence of monocytes to the plastic surface. All incubations were carried out in a humidified incubator at 37°C and 6% CO_2.

Cytokine measurement by flow cytometry

Single cell measurement of intracellular cytokines by flow cytometry was performed essentially as described by Girndt *et al.* [15]. In brief, stimulated cells were fixed using 4% paraformaldehyde (Merck, Darmstadt, Germany) for 5 min and subsequently washed with an excess of ice-cold phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA, Sigma), 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (fluorescence-activated cell sorting (FACS) buffer). To allow antibodies access into the cells, membranes were reversibly permeabilized with 0.1% saponin (Sigma) in FACS buffer containing 5% low-fat milk powder (permeabilization buffer). For staining of IL-12, non-specific binding of cytokine specific antibodies to Fc-receptors on monocytes was blocked by 30 min preincubation using a mixture of human (Cytoglobin, Biotest, Dreieich, Germany) and mouse (Dianova, Hamburg, Germany) immunoglobulin. Cell surface markers and intracellular cytokines were labelled with mouse anti-human cytokine antibodies conjugated to fluorescent dyes at saturating concentrations in permeabilization buffer. For the staining of lymphocytes we used PerCP-labelled anti-CD4 (clone SK3, Becton Dickinson, Heidelberg, Germany), FITC-labelled anti-IFNγ (clone 4S.B3, Pharmingen, Hamburg, Germany), PE-labelled anti-IL-4 (clone 8D4–8, Pharmingen), FITC-labelled anti-CD45RA (clone HI100, Pharmingen) and PE-labelled anti-CD45 RO (clone UCHL1, Pharmingen). Monocytes were stained using PE-labelled anti-IL-12p40 (clone C11.5, Pharmingen). After 45 min of incubation at 4°C cells were washed three times in permeabilization buffer and twice in FACS buffer. Subsequently, stained antigens were fixed with 1% paraformaldehyde.

Measurements were performed on a Becton Dickinson FACScan flowcytometer and the Cellquest software system. Further analysis of data was done on a personal computer with the WinMDI 2.8 system provided by J. Trotter (Scripps Research Institute, La Jolla, CA).

Cytokine measurement in culture supernatant

IFNγ and IL-12p70 secretion from 2×10^6 PBMC/ml was determined in 24 and 12 h culture supernatants, respectively, using standard protocols for sandwich enzyme linked immunoadsorbant assay (ELISA) (IFNγ, IL-12p70, OptEIA, Pharmingen), according to the manufacturer's instructions. The lower limit of cytokine detection is 7.8 pg/ml for both IFNγ and IL-12p70.

Statistical analysis

For data management and statistical analysis we used the Prism V3.0 statistical software (Graphpad, San Diego, USA). All data are given as means $\pm$ standard deviation unless indicated differently. Significance of differences between patients and controls was calculated using the Mann–Whitney test or the unpaired *t*-test with Welch's correction.

Results

HD patients exhibit an increased percentage of Th1 cells compared with healthy controls

To show whether the reduced humoral immune response in HD patients is reflected by a bias of Th cell differentiation we polyclonally stimulated PBMC from patients and controls with PHA in the presence of autologous accessory cells. IL-2 and IFNγ secretion was measured in the supernatant of stimulated cells using ELISA. In line with a reduced activation of T cells [4] we found a lower secretion of IFNγ (Figure 1) as well as a decreased level of IL-2 (data not shown) in supernatants of PBMC from HD patients compared with healthy controls. Although cytokine secretion of controls scatters broadly, it is in marked contrast to levels of HD patients lying close to or below detection limits in 50% of the cases.

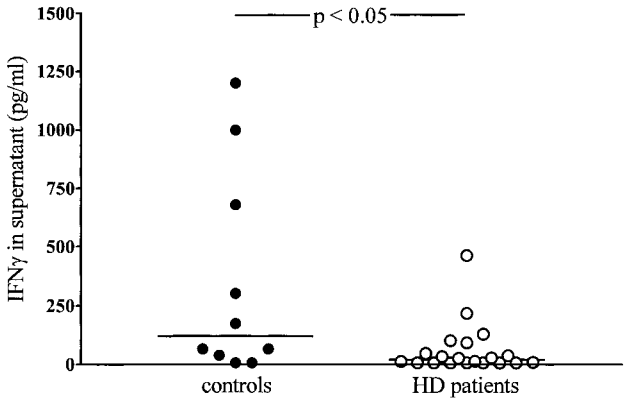

Fig. 1. Measurement by ELISA of IFNγ secretion in supernatant of PBMC stimulated with PHA (62.5 ng/ml) in the presence of autologous monocytes. IFNγ is given as pg/ml per 2×10^6 PBMC/ml after 24 h of stimulation and the median is indicated. Much higher levels are found in supernatants of cells from healthy controls than patients (control subjects *vs* patients, $P < 0.05$). Moreover, $\sim$50% of HD patients exhibit secretion levels below or close to the detection limit. In marked contrast, $\sim$80% of controls show clearly detectable IFNγ levels.

Th1 and Th2 CD4 cells are most reliably distinguished from each other by their cytokine secretion pattern, in that production of IFNγ corresponds to a Th1 and production of IL-4 reveals a Th2 type of Th cell [13]. Among PBMC, several other cell types in addition to CD4-positive T cells are able to secrete IFNγ, therefore the measurement of this cytokine from supernatants by ELISA is not appropriate to define the Th cell pattern. One needs to separately assess the cytokine expression of individual CD4 T cells, which can be done by flow cytometry. However, for this purpose, stimulation with PHA alone is not appropriate to induce sufficient cytokine expression and to reveal the respective differentiation-state. Moreover, due to the reduced activation-state of T cells in HD patients one has to apply stimuli that are independent of co-stimulation and antigen recognition. Therefore, cells are restimulated with PMA and ionomycin after primary stimulation *in vitro* with PHA. We first analysed whether this method was applicable for studying the Th cell differentiation-state of CD4 T cells from HD patients. Cells that were mock-treated did not show any induction of cytokines (data not shown), indicating that a polyclonal stimulation is required to reveal the respective differentiation state. A dotplot showing IFNγ and IL-4 expression of CD4-positive T cells from a control subject following stimulation with PMA and ionomycin is depicted in Figure 2A. In this example, 16.8% of CD4 T cells exhibit a Th1 phenotype (IFNγ) whereas a lower percentage exhibits the Th2 type (IL-4, 3.9%). Th cell differentiation could also clearly be detected in CD4 T cells from HD patients. Figure 2B shows an example with Th1 (24.8%) and Th2 cells (4.2%).

We used this experimental approach to analyse the Th cell pattern of 10 controls and 20 HD patients. Interestingly, in HD patients, a significantly higher percentage of CD4 cells is characterized by a Th1-type cytokine secretion pattern compared with healthy controls (controls $14.7 \pm 4.6\%$ *vs* patients $25.3 \pm 9.7\%$; $P < 0.01$; Figure 3A). Unlike Th1 cells, the percentage of Th2 cells does not differ between patients and controls (controls $6.3 \pm 3.0\%$ *vs* patients $6.3 \pm 3.1\%$; Figure 3B).

Taken together, these data demonstrate that Th cells from HD patients manifest a dysregulated differentiation profile characterized by a major increase in the number of Th1 cells and by a normal amount of Th2 cells.

One could argue that the difference between the two Th cell patterns could be due to an unbalanced ratio of memory *vs* naive CD4 positive cells in patients and controls. We therefore determined this ratio by simultaneously staining naive and memory cells with antibodies directed against CD45RA and CD45RO, respectively. Controls show a similar percentage of CD45RA cells to HD patients (controls $33.1 \pm 10.0\%$ *vs* patients $28.8 \pm 12.8\%$; Figure 4). Thus, we can exclude that a difference in the ratio of naive and memory Th cells between patients and controls accounts for the differentiation profile observed.

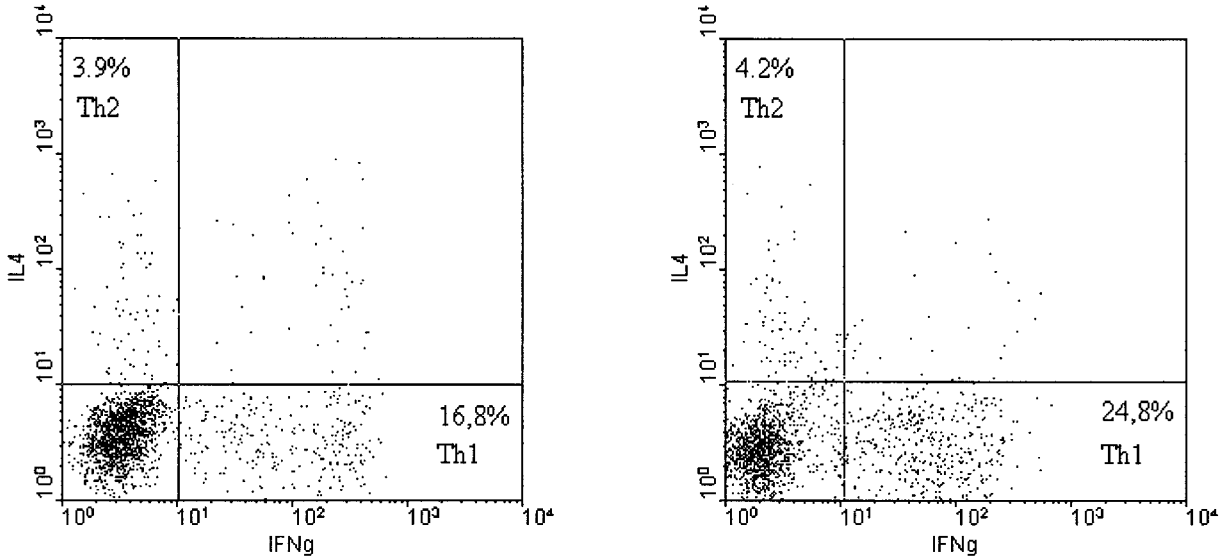

Fig. 2. Dotplots showing the IL-4 (*y*-axis) and IFNγ (*x*-axis) secretion pattern of CD4-positive T cells from a control (**A**) and a HD patient (**B**) after restimulation with 20 ng/ml PMA and 1 μM ionomycin. Percentages give the portion of single positive cells above the cutoff for the respective cytokine. The logarithmic axes of the panels give the fluorescence intensity values assigned to every single cell detected.

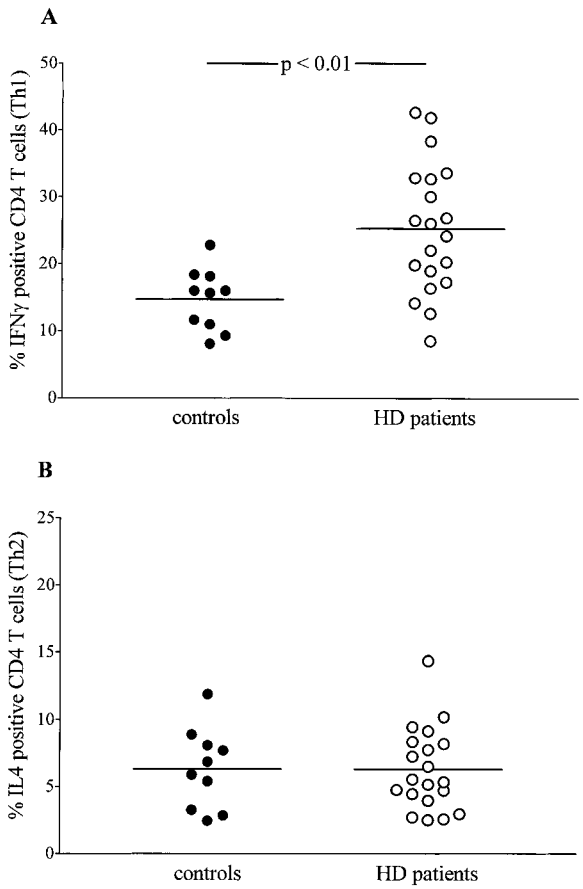

Fig. 3. Percentage of IFNγ (**A**) and IL-4 (**B**) single positive Th cells in patients compared with healthy controls. Bars indicate the mean percentage of Th1 or Th2 cells. The bias towards Th1 seen in HD patients cannot be explained by the difference in age of the two groups tested, since there is no correlation between Th1 cell number and age, neither among HD patients nor in the control group (data not shown).

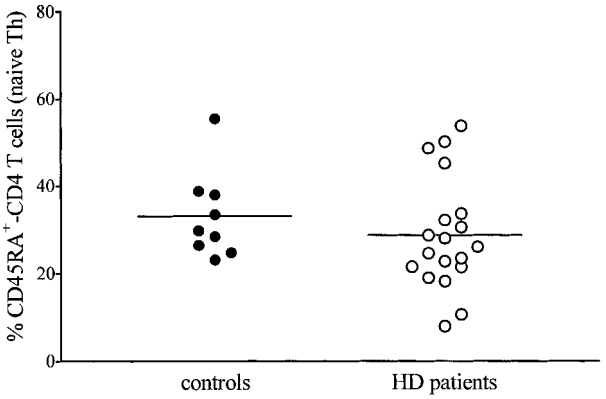

Fig. 4. Percentage of CD45RA positive (naive) CD4 T cells in patients compared with healthy controls. No significant difference is observed between the two groups since controls show a similar percentage of CD45RA cells to HD patients (mean percentage: $33.1 \pm 10.0\%$ *vs* $28.8 \pm 12.8\%$).

HD patients exhibit an increased IL-12 secretion compared with healthy controls

IFNγ and IL-12 are thought to be the major cytokines for promoting Th1 differentiation. The active form of IL-12 (IL-12p70) is secreted by APCs and exists as a heterodimer consisting of IL-12p40 and IL-12p35 [16]. The presence of IL-12 during priming directly augments Th1 differentiation. Interestingly, unlike IFNγ, IL-12 has no effect on Th2 development [17]. Therefore the higher frequency of Th1 cells in HD patients could be explained by a higher level of IL-12, which could facilitate and enhance Th1 differentiation without affecting Th2 development. We tested this hypothesis by comparing the capacity of monocytes from patients and controls to secrete IL-12p70 after priming with IFNγ and stimulation with LPS, which represents a common mode of IL-12 induction. As shown by

ELISA, HD patients exhibit a higher level of IL-12p70 secretion compared with controls (controls 427 ± 264 pg/ml *vs* patients 1040 ± 620 pg/ml; $P = 0.013$; Figure 5A). Using flow cytometry analysis of cytokine production at the single cell level our results were confirmed. These results were extended in that the higher level of IL-12 in HD patients is due to an increased percentage of IL-12-producing monocytes (controls $25.4 \pm 15.2\%$ *vs* patients $48.1 \pm 19.3\%$; $P = 0.017$; Figure 5B), while the level of cytokine secretion per single cell does not differ between patients and controls. We suggest therefore that a higher level of IL-12 favours a skewing of the Th cell response towards Th1.

A bias towards Th1 type CD4 cells seems to exist in vivo

In the experiments described so far, the Th cell profile was analysed after an initial co-stimulation-dependent activation with PHA, propagation with IL-2 and restimulation with PMA/ionomycin after 8 days of culture. This approach bears several advantages, including uniform stimulation conditions, synchronized growth of cells and proliferative enhancement of responding cells. However, one cannot exclude *in vitro* effects such as non-uniform proliferation capacities or various factors imposed by long culture periods.

In an attempt to assess the Th type of cytokine pattern in an assay that most closely reflects the situation found *in vivo* we analysed freshly isolated PBMC directly after polyclonal stimulation with PMA/ionomycin. Although the total percentage of cytokine-positive cells was lower than in the restimulation assays ($15.7 \pm 9.8\%$ *vs* $34.4 \pm 13.1\%$; $P < 0.0001$), we nevertheless found significantly more IFNγ positive cells in HD patients than in healthy controls (controls $8.7 \pm 4.2\%$ *vs* patients $14.5 \pm 9.3\%$; $P = 0.026$; Figure 6A). As shown after restimulation, both groups exhibit equal levels of IL-4-producing cells (controls $2.1 \pm 1.3\%$ *vs* patients $2.0 \pm 1.1\%$; Figure 6B). These results demonstrate that the bias towards Th1-type CD4 cells in HD patients is not due to long term culture conditions but closely reflects the situation found *in vivo*.

Discussion

In order to generate an effective immune response against different pathogens *in vivo*, the organism has

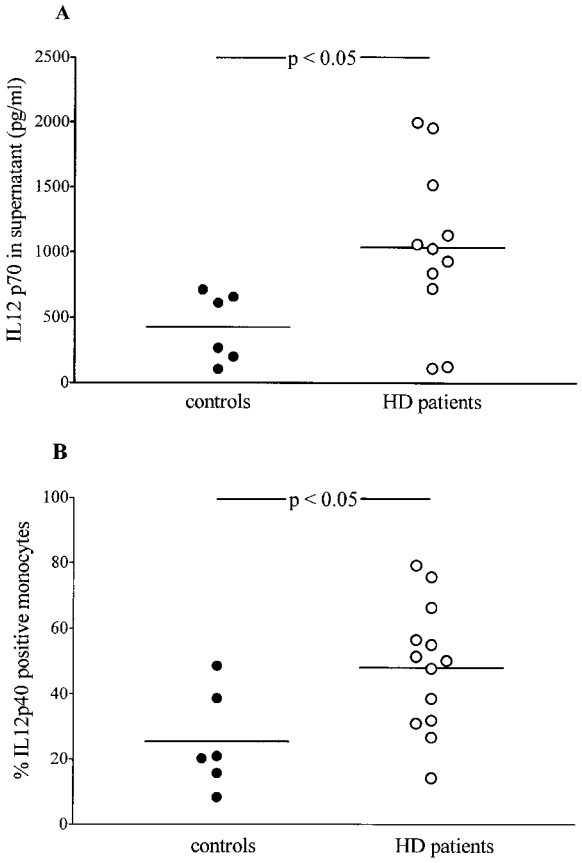

Fig. 5. (**A**) Measurement by ELISA of IL-12p70 secretion in the supernatant of PBMC stimulated with 1000 U/ml IFNγ for 2 h followed by 1 μg/ml LPS for an additional 10 h. IL-12p70 is given as pg/ml per 2×10^6 PBMC/ml after 12 h of stimulation (control subjects *vs* patients, $P = 0.013$). (**B**) Measurement of IL-12p40 at the single cell level by flow cytometry (control subjects *vs* patients, $P = 0.017$). The respective means are indicated.

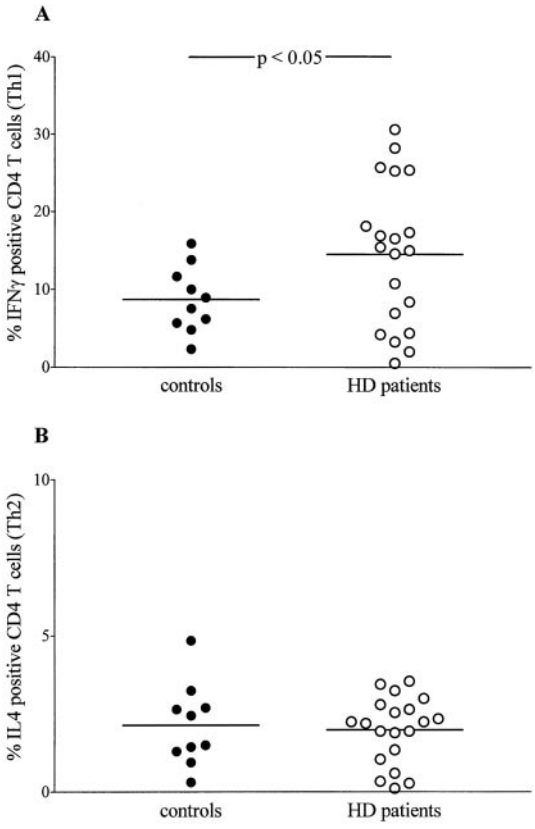

Fig. 6. Percentage of IFNγ (**A**) and IL-4 (**B**) single positive Th cells stimulated with PMA/ionomycin directly after isolation of PBMC from patients and healthy controls. Bars indicate the mean percentage of Th1 or Th2 cells.

to elicit a cellular or humoral immune response, depending on the characteristics of the pathogen. The cellular immune response is supported by Th1 cells and the humoral immune response is mediated by Th2 cells [13]. HD patients show an immunodeficiency that is correlated with a decreased co-stimulatory action of monocytes on T cells *in vitro*. This leads to a reduced IL-2 secretion and proliferation of T cells [4,5], although upregulation of IL-2 receptors indicates the presence of a pre-activated state [18,19]. Furthermore, pro-inflammatory cytokines such as IL-6, TNFα and IL-1β are increased due to a state of chronic inflammation [6,7]. Since both co-stimulation and the cytokine environment are critical factors determining the outcome of Th cell differentiation into either the Th1 or Th2 direction [13,17], we studied the Th cell differentiation of HD patients compared with healthy controls. Although several cell surface molecules, such as chemokine receptors, have been discussed as potential markers for Th1 or Th2 cells [20], IFNγ and IL-4 are the most accurate markers for clearly distinguishing between the two populations. We used flow cytometry to determine them at the single-cell level.

The major finding of our study is that, in the presence of a reduced total activation of T cells, the fraction of CD4 T cells from HD patients that are committed to a Th1 pattern is significantly increased. Upon polyclonal stimulation, our technical approach allows the assessment of the total number of Th cells already committed to a specific differentiation state among all T cells, whereas uncommitted cells do not respond to the applied stimuli. Our finding, together with the observation that the CD4 T-cell population contains normal percentages of Th2 cells, strongly suggests that Th-cell differentiation in HD patients is skewed towards a Th1 direction. This effect is prevalent both in cells after restimulation and in freshly isolated cells, indicating that this biased ratio directly reflects the differentiation state of circulating CD4 cells *in vivo*.

The bias towards Th1 differentiation in HD patients most probably results from an abnormality in the regulation of IL-12, the APC-derived cytokine that is the primary inducer of Th1 cells [21,22]. This is supported by higher levels of IL-12 in supernatants of stimulated monocytes from HD patients. We demonstrated that the production of IL-12 per single cell is not altered, thus leaving two main factors contributing to increased levels of IL-12. One factor is our observation, as well as that of others, that HD patients have increased numbers of circulating monocytes [23]. Secondly, we show that among these patients a higher percentage is capable of producing IL-12. This overproduction of IL-12 is reminiscent of the expression of other pro-inflammatory cytokines found to be dysregulated in HD patients, such as IL-6, TNFα and IL-1β [6–8,24,25]. Similar to IL-12, we recently demonstrated that increased IL-6 levels are due to an increased percentage of cytokine-producing cells, whereas the expression per single cell equals that of control cells [15]. Moreover, we previously showed that the overproduction of IL-6 is controlled by the monocyte-derived regulatory cytokine IL-10, in that the patient's ability to upregulate IL-10 critically determines the level of IL-6 expression [7]. It is well established that IL-12 expression is also tightly controlled by IL-10 [26]. Consequently, aberrant production of the Th1-inducing cytokine IL-12 may be induced by similar regulatory mechanisms as shown for IL-6.

We suggest a model that conceivably incorporates characteristic features found to correlate with impaired immune responses in HD patients, namely the decreased co-stimulatory activity of monocytes and a higher expression level of pro-inflammatory cytokines. A decreased co-stimulatory activity of monocytes leads to a general reduction in T-cell activation, both Th1 and Th2. An increased level of IL-12 leads to a bias in the differentiation of Th cells towards Th1 whereas the number of Th2 cells is not affected. Thus, one could imagine that the negative effect of a decreased proliferation of T cells could be partially compensated by the IL-12 induced increase of Th1 cell numbers. In contrast, relative Th2 cell numbers are normal.

In recent years, studies of the pathogenesis of various autoimmune diseases have shown that inflammatory lesions occurring in some of these diseases are driven by T cells that display either a biased Th1 or Th2 type of Th cell differentiation. For example, WG is associated with an unbalanced Th1 ratio [10], whereas SLE is dominated by T cells exhibiting a Th2-type pattern [11]. Interestingly, during the course of progression to end-stage renal failure, one clinically observes a remission of SLE, whereas WG generally is not remedied. These observations correlate nicely with our findings of an imbalance of Th cell profiles towards Th1 in HD patients. Thus, in the case of SLE, both the shift towards Th1 cell differentiation and the decreased co-stimulation could result in a remission of autoimmune disease in the course of the progression to chronic renal failure. In the case of WG, however, no remission is observed during chronic renal failure progression, presumably since a bias towards Th1 is sustained.

To analyse whether this Th1 shift does indeed correlate with disease remission during the progress of chronic renal failure, one could prospectively follow the bias of Th cell differentiation in a longitudinal study in patients with various autoimmune diseases during the course of renal disease. We are currently investigating whether this concept holds true.

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