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ORIGINAL ARTICLE

Observational support for an immunoregulatory role of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ blood lymphocytes in kidney transplant recipients with good long-term graft outcome

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Summary

There is evidence that interferon-gamma (IFN-γ)-dependent interactions of dendritic cell (DC), T regulatory (Treg), and T suppressor (Ts) subpopulations contribute to allograft acceptance. We measured DC subsets, $CD3^+CD4^+CD25^+$ (Treg phenotype) and $CD3^+CD8^+CD28^-$ (Ts phenotype) peripheral blood lymphocytes (PBL) expressing Foxp3, Th1 or Th2 cytokines, peripheral T- and B-cell counts, and plasma cytokines in 33 kidney transplant recipients with a serum creatinine of ≤1.8 mg/dl and 32 recipients with a serum creatinine of ≥2.0 mg/dl more than 100 days post-transplant. Cell subsets were measured in whole blood using four-color flow cytometry. Patients with increased creatinine had less frequently detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL than patients with good graft function ($P = 0.017$). In patients with good graft function, $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL were associated with high $Foxp3^+$, IL-2^+, IL-12^+, IL-4^+, and IL-10^+ $CD3^+CD4^+CD25^+$ T PBL ($P < 0.001$), low $CD3^+CD8^+CD28^-Foxp3^+$ ($P = 0.002$), $CD3^+CD4^+DR^+$ ($P = 0.002$), $CD3^+CD8^+DR^+$ T ($P = 0.005$) and $CD19^+$ B PBL ($P = 0.005$), and low $lineage^-HLA\text{-}DR^+CD11c^+CD123^-$ DC1 ($P = 0.006$). Patients with impaired graft function did not show these associations. Additional flow cytometric analysis confirmed strong co-expression of IFN-γ and Foxp3 by $CD4^+CD25^+$ PBL particularly in patients with good graft function. Our data support an immunoregulatory role of $CD3^+CD4^+CD25^+Foxp3^+IFN\text{-}\gamma^+$ cells in a subgroup of transplant recipients with good graft acceptance.

Introduction

Transplant recipients with functioning grafts were shown to possess increased circulating $lineage^-HLA\text{-}DR^+CD11c^-CD123^+$ plasmacytoid dendritic cells type 2 (DC2) precursors, forkehead/winged helix transcription factor (Foxp3)-expressing $CD3^+CD4^+CD25^+$ regulatory T cells (Treg), $CD3^+CD8^+CD28^-$ T-suppressor lymphocytes (Ts), TGF-$ß_2$-producing T-helper lymphocytes type 3 (Th3), $TCR^+CD4^-CD8^-$ T, and NK Treg cells. However, the contribution of these cell subsets to the establishment and maintenance of graft acceptance is unclear [1–3]. Treg and Ts were shown to produce interleukin (IL)-10 but not interferon-gamma (IFN-γ) and it was suggested that IL-10 might in part account for the immunosuppressive effect of Treg and Ts [2–4].

Recently, we described in long-term stable kidney transplant recipients a domination of myeloid IL-10-producing DC [5]. Contrary to our expectation, the patients showed abnormally high IFN-γ and IL-2, but low IL-10 plasma levels. More than 10 years post-transplant, some patients showed IFN-γ plasma levels three-times higher than healthy controls [5]. We confirmed these data in another study and speculated that the abnormally increased IFN-γ

plasma levels might be associated with IFN-γ-producing Treg and might contribute to graft acceptance [6]. Meanwhile, there is additional support for that hypothesis. Sawitzki *et al.* [7] described in CD4$^+$CD25$^+$ T cells, but not in CD4$^+$CD25$^-$ T cells of mice, a fivefold increase in IFN-γ mRNA expression within 24 h of re-encountering alloantigen *in vivo*, and an associated increase of IFN-γ protein in the supernatant of Treg cell cultures. *In vivo* neutralization of IFN-γ with anti-IFN-γ monoclonal antibody resulted in necrosis of skin grafts [7]. Wood and Sawitzki [8] reported recently that IFN-γ, produced rapidly and only transiently by induced Treg, can inhibit the activation and proliferation of IFN-γR1- and IFN-γR2-bearing T cells. Because the allogeneic stimulus in transplant recipients is permanent, we find it conceivable that patients with well-functioning grafts have permanently activated Treg lymphocytes that continuously produce IFN-γ. IFN-γ-producing Treg might be associated with increased IFN-γ plasma levels in patients with good graft outcome, which would support the concept that IFN-γ-mediated immune mechanisms contribute to long-term graft acceptance in kidney transplant recipients.

In the present study, we investigated whether there is an association of CD3$^+$CD4$^+$CD25$^+$IFN-γ$^+$ peripheral blood lymphocytes (PBL) with IFN-γ plasma levels and whether this association is related to the quality of long-term graft function. We investigated whether CD3$^+$CD4$^+$CD25$^+$IFN-γ$^+$ PBL express, in addition, Foxp3, which would suggest that CD3$^+$CD4$^+$CD25$^+$IFN-γ$^+$Foxp3$^+$ PBL might have an immunoregulatory role. We tried to find additional indications for an immunoregulatory role of CD3$^+$CD4$^+$CD25$^+$IFN-γ$^+$ PBL by looking for possible associations of CD3$^+$CD4$^+$CD25$^+$IFN-γ$^+$ PBL with activated CD3$^+$CD8$^+$DR$^+$ T cells, T cells with Ts phenotype, T cells with altered IL-7 receptor expression, CD19$^+$ B cells, CD3$^-$CD16$^+$ NK cells, DC1 and DC2 subsets, and other plasma cytokines. This study represents an attempt of examining a possible immunoregulatory role of CD3$^+$CD4$^+$CD25$^+$IFN-γ$^+$ PBL in the complex network of cellular and humoral interactions *in vivo*.

Materials and methods

Patients and healthy controls

We studied 65 kidney transplant recipients from the outpatient clinic. Thirty-three patients had good kidney function and a serum creatinine of ≤1.8 mg/dl whereas 32 patients had impaired kidney function with a serum creatinine of ≥2.0 mg/dl. All patients were more than 100 days post-transplant (mean ± SD: 1717 ± 1580 days vs. 1744 ± 1391 days; P = NS) (Table 1). Based on laboratory tests and clinical examination at the time of investigation, none of the patients showed evidence of acute rejection or infection. Demographic data of the patient groups are shown in Table 1. Sixty-three healthy regular blood donors served as controls. The mean age of the healthy controls was 28.5 ± 8.2 years (range: 18–50 years). Thirty-eight healthy controls were male. All patients and controls gave informed consent for the tests performed under this study and the study was approved by the local ethical committee. The study was conducted in adherence to the Declaration of Helsinki.

Determination of lineage$^-$HLA-DR$^+$CD11c$^+$CD123$^-$ DC1 and lineage$^-$HLA-DR$^+$CD11c$^-$CD123$^+$ DC2

Preparation of assays

Myeloid DC1 and plasmacytoid DC2 precursors were determined in freshly obtained heparinized whole blood using four-color fluorescence flow cytometry. Twenty microliter FITC-, PE-, or PE-Cy5-labeled mouse-IgG1 (BD, Heidelberg, Germany) and 20 µl PE- or APC-coupled mouse-IgG2b were used as isotype controls and were pipetted into tube A. Tube B contained monoclonal antibodies against lineage (10 µl CD3/PE, 10 µl CD14/PE, 10 µl CD16/PE, 10 µl CD19/PE, BD), CD11c (APC, 5 µl, BD), HLA-DR, DP, DQ (FITC, 20 µl, BD), and CD123 (PE-Cy5, 20 µl, BD). A quantity of 100 µl of freshly obtained heparinized whole blood was added to each tube and incubated for 30 min at 22 °C. A quantity of 2 ml FACS lysing solution (BD, diluted 1:10 with aqua dest.) was pipetted into each tube, incubated for 10 min, and centrifuged at 200 *g* for 8 min. The supernatant was removed, 2 ml phosphate-buffered saline (PBS) (Gibco Invitrogen, Karlsruhe, Germany) containing 0.1% NaN_3 and 1% fetal calf serum was added, and the tubes were centrifuged again. A quantity of 500 µl PBS was added to each tube and the assays were analyzed immediately using a FACScalibur flow cytometer (BD).

Gating procedure

At least 100 000 events were analyzed. In a first step, lineage$^-$ but HLA-(DR, DP, DQ)$^+$ cells were gated and that subset was further investigated in a second gating step for CD11c$^+$ and/or CD123$^+$ cells. CD11c$^+$CD123$^-$ cells were designated as DC1 and CD11c$^-$CD123$^+$ cells as DC2.

Determination of CD3$^+$CD4$^+$CD25$^+$cytokine$^+$ and CD3$^+$CD8$^+$CD28$^-$cytokine$^+$ PBL

Preparation of assay

CD3$^+$CD4$^+$CD25$^+$cytokine$^+$ and CD3$^+$CD8$^+$CD28$^-$cytokine$^+$ lymphocytes were determined in freshly obtained heparinized whole blood using four-color fluorescence flow cytometry. Isotype control (tube 1) consisted of each 20 µl FITC-, PE-, PerCP- or APC-coupled mouse- and

Table 1. Demographic data of renal transplant recipients with good (crea ≤ 1.8 mg/dl) or impaired (crea ≥ 2.0 mg/dl) graft function.

Parameter	Good graft function (n = 33)	Impaired graft function (n = 32)	P-value
Serum creatinine (mg/dl; mean ± SD; range)	1.4 ± 0.3 (0.7–1.8)	2.7 ± 0.7 (2.0–4.9)	**<0.001**
Post-transplant interval (days; mean ± SD; range)	1717 ± 1580 (118–6521)	1744 ± 1391 (107–5762)	0.773
Age (years; mean ± SD; range)	53 ± 12 (28–73)	51 ± 17 (21–76)	0.906
Living related/deceased donor (n)	10/23	12/20	0.540
Female/male (n)	14/19	11/21	0.505
Panel reactive antibodies >5% (n)	5	5	0.958
HLA-mismatch (A, B, DR; mean ± SD; range)	2.4 ± 1.7 (0–5)	2.8 ± 1.5 (0–6)	0.404
HLA-match (A, B, DR; mean ± SD; range)	3.3 ± 1.6 (0–6)	2.9 ± 1.4 (0–6)	0.463
Blood cyclosporine level (ng/ml; mean ± SD; range)	157 ± 43 (103–228)	148 ± 19 (121–175)	0.842
Blood tacrolimus level (ng/ml; mean ± SD; range)	6.6 ± 2.0 (3–10)	6.2 ± 1.7 (4–11)	0.351
Cyclosporine (mg/kg/day)	2.93 ± 0.68 (n = 11)	2.43 ± 0.66 (n = 10)	0.173
Prednisolone (mg/kg/day)	0.09 ± 0.05 (n = 19)	0.11 ± 0.06 (n = 26)	0.151
Mycophenolate mofetil (mg/kg/day)	17 ± 7.2 (n = 15)	18 ± 9.1 (n = 15)	0.713
Azathioprine (mg/kg/day)	1.1 ± 0.42 (n = 10)	0.90 ± 0.34 (n = 5)	0.594
Tacrolimus (mg/kg/day)	0.05 ± 0.05 (n = 21)	0.04 ± 0.02 (n = 19)	0.421
Everolimus (mg/kg/day)	0.02 (n = 1)	0.02 (n = 1)	–
Sirolimus (mg/kg/day)	0.05 (n = 1)	0.03 ± 0.01 (n = 4)	0.400
ATG induction treatment (n)	5	6	0.699
Daclizumab (n)	3	2	0.667
Simulect (n)	6	6	0.953
Diagnosis chronic allograft nephropathy (n)	0	5	0.018
Acute rejection during the first year post-transplant (n)	4	5	0.683
Severe infection during the last year before investigation (n)	4	6	0.459

Adjustment for multiple testing was made according to the method of Bonferroni.
P-values < 0.01 are considered significant and are bold printed in the tables.

rat-IgG1 (BD). For the determination of $CD3^+CD4^+CD25^+$ (tubes 2–7) and $CD3^+CD8^+CD28^-$ lymphocytes (tubes 8–13) the monoclonal antibodies CD3/FITC (20 µl, BD), CD4/PerCP (20 µl, BD), CD8/PerCP (20 µl, BD), CD25/APC (5 µl, BD), and CD28/APC (20 µl, BD) were used. A quantity of 100 µl of freshly obtained heparinized whole blood was added to each tube, and the tubes were vortexed and incubated for 30 min at 22 °C in the dark. Thereafter, 2 ml FACS lysing solution (BD, diluted 1:10 with aqua dest.) was pipetted into each tube, the tubes were vortexed, incubated for 10 min at room temperature in the dark and centrifuged at 200 *g* for 8 min. The supernatant was removed, 2 ml PBS (Gibco) containing 0.1% NaN_3 and 1% fetal calf serum was added, and the tubes were centrifuged at 200 *g* for 8 min. The supernatant was removed and the cell membranes were permeabilized by adding 500 µl Perm2 solution (BD, diluted 1:10 with aqua dest.). The tubes were incubated for 10 min at 22 °C in the dark, 2 ml of PBS was added, and the tubes were centrifuged at 200 *g* for 8 min. The supernatant was removed and the monoclonal antibodies IL-2/PE (20 µl, BD, tubes 2 + 8), IL-12/PE (20 µl, BD, tubes 3 + 9), IFN-γ/PE (20 µl, BD, tubes 4 + 10), IL-4/PE (5 µl of a 1:100 dilution, BD, tubes 5 + 11), and IL-10/PE (5 µl of a 1:100 dilution, BD, tubes 6 + 12) were added. Intracellular Foxp3 expression was determined using Foxp3/PE (20 µl, tubes 7 + 13; NatuTec, Frankfurt, Germany) monoclonal antibody. After incubation for another 30 min at 22 °C in the dark, 2 ml PBS was added to the tubes, the tubes were centrifuged at 200 *g* for 8 min, and the supernatant was removed. Five-hundred microliter of PBS was added to each tube and the assays were analyzed immediately using a FACScalibur flow cytometer (BD).

Gating procedures

At least 100 000 events were analyzed. Initially, $CD3^+CD4^+$ or, alternatively, $CD3^+CD8^+$ lymphocytes were gated. Within the $CD3^+CD4^+$ cell fraction, $CD25^+$cytokine$^+$ lymphocytes were determined, whereas within the $CD3^+CD8^+$ cell fraction, $CD28^-$cytokine$^+$ lymphocytes were studied. In addition, $CD3^+CD4^+CD25^+Foxp3^+$ and $CD3^+CD8^+CD28^-Foxp3^+$ lymphocytes were determined.

Determination of plasma cytokines, soluble cytokine receptors, and soluble cytokine receptor antagonists

Plasma soluble interleukin-1 receptor antagonist (sIL-1RA), IL-2, soluble IL-2 receptor (sIL-2R), IL-3, IL-4, IL-6, sIL-6R, IL-10, transforming-growth-factor-β_2 (TGF-β_2), IFN-γ, and tumor necrosis factor alpha (TNF-α) were determined by ELISA. sIL-1RA, IL-2, sIL-2R, IL-3, IL-4,

IL-6, sIL-6R, IL-10, TGF-β_2, and TNF-α were measured using Quantikine kits (R&D Systems, Wiesbaden, Germany), and IFN-γ using HBT kits (Holland biotechnology BV; Biermann, Bad Nauheim, Germany). Plasma was snap-frozen within 2 h after the blood was drawn and stored at −30 °C until testing. We have used this cytokine panel for cytokine monitoring of renal transplant recipients since 1995 [9].

Plasma neopterin

Plasma neopterin was measured with the Neopterin-ELItest assay (Brahms Diagnostics, Berlin, Germany). Based on control measurements in 70 healthy individuals, a plasma neopterin level greater than 15 nmol/l was considered abnormally high.

Determination of lymphocyte subpopulations

CD3$^+$CD16$^-$CD19$^-$, CD3$^-$CD16$^+$CD19$^-$, CD3$^-$CD16$^-$CD19$^+$, CD3$^+$CD4$^+$CD8$^-$, CD3$^+$CD4$^-$CD8$^+$, CD3$^+$CD4$^+$DR$^+$, CD3$^+$CD4$^-$DR$^+$ (corresponding to CD3$^+$CD8$^+$DR$^+$), CD3$^+$CD25$^+$, CD3$^+$CD4$^+$CD127$^+$, and CD3$^+$CD8$^+$CD127$^+$ lymphocyte subsets were defined using triple-fluorescence flow cytometry. As isotype controls served IgG2a/FITC, IgG2a/PE, and IgG2a/Cy-Chrome antibodies. All antibodies were purchased from Becton Dickinson/Pharmingen (BD). A quantity of 10 µl of a mixture of three different monoclonal antibodies conjugated with FITC, PE, or Cy-Chrome were added to 50 µl of heparinized whole blood and incubated for 15 min at 4 °C. Erythrocytes were lysed with NH_4Cl for 15 min. Lymphocyte subsets were analyzed using an Ortho Cytoron Absolute flow cytometer (Ortho Clinical Diagnostics, Heidelberg, Germany). The flow cytometer was calibrated every day using the Ortho calibration kit (Hiss Diagnostics, Freiburg, Germany) to ensure optimal counting.

Statistical analysis

Wilcoxon rank sum test, Spearman's rank correlation test, and Fisher's exact test were used for statistical analysis applying the Statistical Program for the Social Sciences (SPSS, Chicago, IL, USA). Adjustment for multiple testing was done according to the method of Bonferroni. *P*-values of <0.01 were considered significant and are boldprinted in the tables.

Results

Occurrence of CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL

CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL were detectable in 76% of patients with good graft outcome and 65% of healthy controls (P = NS), but only in 47% of transplant recipients with increased serum creatinine late post-transplant (compared to patients with good graft function: $P = 0.017$) (Fig. 1). Patients with good graft function had both higher relative- and higher absolute counts of CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL than patients with impaired graft function ($P = 0.019$; $P = 0.015$) (Table 2). Lower CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL, and higher plasma levels of neopterin ($P < 0.001$) and IL-6 ($P = 0.013$) in patients with impaired graft function were the only immune parameters of a total of 64 investigated immune parameters that were different between patients with good and patients with impaired graft function (Table 2). Both patient groups showed similar differences in distinct immune parameters in comparison to healthy controls and these differences might be mainly attributed to the immunosuppressive drugs that the patients in both groups received (Table 2).

Immunosuppressive protocol, CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ and CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ PBL, and IFN-γ plasma levels

Cyclosporine and tacrolimus blood levels, daily doses of cyclosporine, tacrolimus, prednisolone, mycophenolate mofetil, and azathioprine as well as frequency of induction therapy with antithymocyte globulin (ATG), daclizumab, or simulect were similar in the two patient groups (P = NS) (Table 1). Drug doses and blood levels of the immunosuppressive drugs were neither associated with relative or absolute numbers of CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ and CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ PBL nor with IFN-γ plasma levels and IFN-γ/serum creatinine ratios in patients with good (P = NS) or patients with impaired graft function (P = NS). Twenty-eight patients received induction treatment with poly- or monoclonal antibodies. Prophylactic antibody treatment did not affect the occurrence of CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL late post-transplant. A total of seven out of 11 patients treated with ATG, two out of five patients treated with daclizumab, and nine out of 12 patients treated with simulect had detectable CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL (P = NS). The frequencies of CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL were similar in antibody-treated patients both with good or impaired graft function (P = NS) (data not shown). Similar results were observed for absolute numbers of CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ PBL and IFN-γ plasma levels or IFN-γ/serum creatinine ratios in patients with good (P = NS) or impaired graft function (P = NS). The data suggest that the two patient groups received similar immunosuppressive protocols and that differences in the doses of the immunosuppressive drugs late post-transplant were not associated with differences in CD3$^+$CD4$^+$CD25$^+$IFN-γ^+

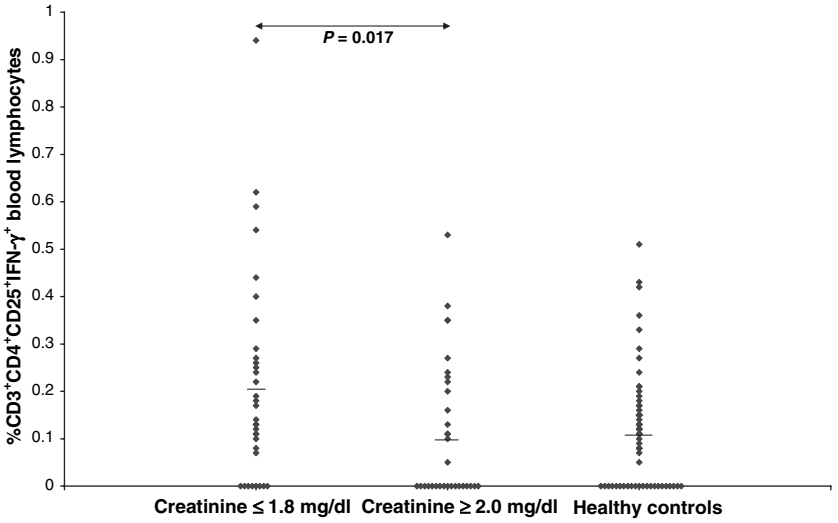

Figure 1 Proportions of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL in 33 kidney transplant recipients with good graft function (creatinine ≤ 1.8 mg/dl), 32 transplant recipients with impaired graft function (creatinine ≥ 2.0 mg/dl), and 63 healthy controls. Only 15 (47%) patients with impaired graft function had detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL, in contrast to 25 (76%) patients with good graft function ($P = 0.017$), and 41 (65%) healthy controls. Mean values are indicated by bars.

and $CD3^+CD4^+CD25^+Foxp3^+$ PBL, or IFN-γ plasma levels.

Associations of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL with $CD3^+CD4^+CD25^+Foxp3^+$ PBL and further immune parameters

Usually, $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ represents the phenotype of activated-Th1 PBL. Because patients with good graft function showed higher $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL than patients with impaired graft function, we hypothesized that $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL might have immunosuppressive rather than immunostimulatory function. In order to examine this hypothesis, we tried to find associations of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL with $CD3^+CD4^+CD25^+Foxp3^+$ PBL and further immune parameters listed in Table 2.

Patients with good graft function showed a strong association of relative numbers of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL with high relative (and absolute) numbers of $CD3^+CD4^+CD25^+$ PBL expressing Foxp3 ($P < 0.001$) (Fig. 2a), IL-2 ($P < 0.001$), IL-12 ($P < 0.001$), IL-4 ($P < 0.001$), and IL-10 ($P < 0.001$), and high $CD127^+$ $CD3^+CD4^+$ PBL ($P = 0.003$) suggesting that these proteins might be expressed and up-regulated by the same cell or, alternatively, in parallel by different cells (Table 3). Furthermore, patients with good graft function showed an association of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL proportions with low absolute numbers of Foxp3-expressing $CD3^+CD8^+CD28^-$ PBL representing PBL with Ts phenotype ($P = 0.002$), low absolute numbers of total lymphocytes ($P = 0.007$), $CD3^+$ ($P = 0.005$), $CD3^+CD8^+$ ($P = 0.001$), $CD3^+DR^+$ ($P < 0.001$), $CD3^+CD4^+DR^+$ ($P = 0.002$), $CD3^+CD8^+DR^+$ ($P = 0.005$), and $CD3^+CD8^+CD127^+$ ($P = 0.007$) T lymphocytes, $CD19^+$ B lymphocytes ($P = 0.005$), DR^+ cells ($P < 0.001$), low proportions of $lineage^-HLA\text{-}DR^+CD11c^+CD123^-$ DC1 ($P = 0.006$), and low levels of plasma IL-3 ($P = 0.001$) (Table 3). When, in addition, associations of $Foxp3^+$ $CD3^+CD4^+CD25^+$ PBL with immune parameters were studied, $Foxp3^+$ $CD3^+CD4^+CD25^+$ PBL showed significant associations with the same immune parameters as $IFN\text{-}\gamma^+$ $CD3^+CD4^+CD25^+$ PBL (versus $CD3^+CD4^+CD25^+$ PBL expressing IL-2 ($P < 0.001$), IL-12 ($P < 0.001$), IL-4 ($P = 0.002$), and IL-10 ($P < 0.001$), $CD3^+CD8^+CD28^-$ $Foxp3^+$ PBL ($P = 0.033$), total lymphocytes ($P = 0.040$), $CD3^+$ ($P = 0.032$), $CD3^+CD8^+$ ($P = 0.060$), $CD3^+DR^+$ ($P = 0.002$), $CD3^+CD4^+DR^+$ ($P = 0.001$), $CD3^+CD8^+DR^+$ ($P = 0.043$) T lymphocytes, $CD19^+$ B lymphocytes ($P = 0.015$), DR^+ cells ($P = 0.001$), $lineage^-HLA\text{-}DR^+CD11c^+CD123^-$ DC1 ($P = 0.010$), and plasma IL-3 ($P = 0.043$)) (Table 3). The data suggest that Foxp3 and IFN-γ might be expressed by the same or by synergistically acting $CD3^+CD4^+CD25^+$ PBL and that these cells are associated with low levels of activated $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, DC1, PBL with Ts phenotype, and plasma IL-3, pointing to an immunosuppressive role of IFN-γ- and/or Foxp3-expressing $CD3^+CD4^+CD25^+$ PBL in patients with good graft function.

In contrast, patients with impaired graft showed an association of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL only with low proportions of DR^+ cells ($P < 0.001$) and, in addition, an association of $CD3^+CD4^+CD25^+Foxp3^+$ PBL only with high proportions of $CD3^+CD25^+$ ($P < 0.001$) and IL-12-producing $CD3^+CD4^+CD25^+$ PBL ($P = 0.006$) (Table 3). The data indicate that $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL in patients with impaired graft function are not associated with $CD3^+CD4^+CD25^+Foxp3^+$ PBL (P = NS) (Fig. 2b) and low levels of T and B PBL, DC1, or plasma

Table 2. DC, $CD3^+CD4^+CD25^+$ and $CD3^+CD8^+CD28^-$ PBL subsets, lymphocyte subpopulations, and plasma cytokines in transplant recipients with good (crea ≤ 1.8 mg/dl) or impaired (crea ≥ 2.0 mg/dl) graft function, and healthy controls.

Parameter	Good graft function (Group 1) (n = 33)	Impaired graft function (Group 2) (n = 32)	Healthy controls (Group 3) (n = 63)	Group 1 vs. 2 P-value	Group 1 vs. 3 P-value	Group 2 vs. 3 P-value
% $CD3^+CD4^+CD25^+IL\text{-}2^+$	0.54 ± 0.59	0.51 ± 0.45	0.30 ± 0.25	0.757	0.086	0.043
$CD3^+CD4^+CD25^+IL\text{-}2^+/\mu l$	3.9 ± 3.8	3.9 ± 4.0	3.0 ± 2.6	0.850	0.328	0.492
% $CD3^+CD4^+CD25^+IL\text{-}4^+$	0.25 ± 0.34	0.23 ± 0.30	0.29 ± 0.52	0.836	0.692	0.965
$CD3^+CD4^+CD25^+IL\text{-}4^+/\mu l$	1.9 ± 2.4	1.8 ± 2.4	2.5 ± 4.2	0.585	0.955	0.658
% $CD3^+CD4^+CD25^+IL\text{-}10^+$	0.24 ± 0.36	0.14 ± 0.21	0.13 ± 0.21	0.235	0.230	0.644
$CD3^+CD4^+CD25^+IL\text{-}10^+/\mu l$	1.7 ± 2.1	0.98 ± 1.9	1.3 ± 1.8	0.124	0.354	0.233
% $CD3^+CD4^+CD25^+IL\text{-}12^+$	0.35 ± 0.40	0.31 ± 0.37	0.22 ± 0.17	0.490	0.441	0.962
$CD3^+CD4^+CD25^+IL\text{-}12^+/\mu l$	2.6 ± 2.7	2.1 ± 3.6	2.1 ± 1.5	0.229	0.843	0.138
% $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$	0.20 ± 0.22	0.10 ± 0.14	0.11 ± 0.12	0.019	0.054	0.304
$CD3^+CD4^+CD25^+IFN\text{-}\gamma^+/\mu l$	1.4 ± 1.3	0.82 ± 1.3	1.2 ± 1.3	0.015	0.203	0.051
% $CD3^+CD4^+CD25^+Foxp3^+$	20 ± 10	20 ± 12	14 ± 4.2	0.722	0.016	0.123
$CD3^+CD4^+CD25^+Foxp3^+/\mu l$	149 ± 81	130 ± 118	144 ± 83	0.083	0.587	0.046
% $CD3^+CD8^+CD28^-IL\text{-}2^+$	0.75 ± 0.70	0.78 ± 0.78	0.56 ± 0.52	0.978	0.297	0.248
$CD3^+CD8^+CD28^-IL\text{-}2^+/\mu l$	4.4 ± 4.6	6.3 ± 8.7	3.0 ± 2.7	0.756	0.300	0.298
% $CD3^+CD8^+CD28^-IL\text{-}4^+$	0.40 ± 0.84	0.40 ± 0.35	0.43 ± 0.89	0.121	0.656	0.056
$CD3^+CD8^+CD28^-IL\text{-}4^+/\mu l$	2.4 ± 5.5	3.2 ± 3.9	2.1 ± 4.7	0.150	0.851	0.090
% $CD3^+CD8^+CD28^-IL\text{-}10^+$	0.20 ± 0.21	0.26 ± 0.25	0.12 ± 0.18	0.333	0.025	**0.002**
$CD3^+CD8^+CD28^-IL\text{-}10^+/\mu l$	1.1 ± 1.1	2.0 ± 3.5	0.64 ± 1.0	0.672	0.024	**0.007**
% $CD3^+CD8^+CD28^-IL\text{-}12^+$	0.44 ± 0.45	0.48 ± 0.45	0.37 ± 0.40	0.690	0.487	0.228
$CD3^+CD8^+CD28^-IL\text{-}12^+/\mu l$	2.3 ± 2.8	3.5 ± 4.7	1.9 ± 2.1	0.465	0.726	0.178
% C $CD3^+CD8^+D28^-IFN\text{-}\gamma^+$	0.36 ± 0.87	0.24 ± 0.37	0.16 ± 0.22	0.653	0.073	0.239
$CD3^+CD8^+CD28^-IFN\text{-}\gamma^+/\mu l$	2.9 ± 8.9	1.9 ± 3.3	0.91 ± 1.3	0.869	0.085	0.201
% $CD3^+CD8^+CD28^-Foxp3^+$	44 ± 21	51 ± 26	29 ± 14	0.156	**0.001**	**<0.001**
$CD3^+CD8^+CD28^-Foxp3^+/\mu l$	261 ± 246	401 ± 424	163 ± 96	0.428	0.110	0.073
% $CD3^+CD4^+CD127^+$	96 ± 5.0	96 ± 4.6	97 ± 2.4	0.958	0.723	0.474
$CD3^+CD4^+CD127^+/\mu l$	769 ± 381	738 ± 437	1015 ± 511	0.667	**0.008**	**0.008**
% $CD3^+CD8^+CD127^+$	68 ± 20	64 ± 23	83 ± 12	0.596	**<0.001**	**<0.001**
$CD3^+CD8^+CD127^+/\mu l$	310 ± 166	417 ± 344	476 ± 182	0.483	**<0.001**	0.014
Plasma neopterin (nmol/l)	13 ± 6.4	49 ± 56	6.8 ± 2.2	**<0.001**	**<0.001**	**<0.001**
Plasma sIL-1RA (pg/ml)	1316 ± 852	1564 ± 2304	228 ± 209	0.163	**<0.001**	**<0.001**
Plasma IL-2 (pg/ml)	10 ± 9.9	12 ± 12	10 ± 8.2	0.634	0.742	0.863
Plasma sIL-2R (pg/ml)	1817 ± 1367	2841 ± 3970	886 ± 322	0.033	**<0.001**	**<0.001**
Plasma IL-3 (pg/ml)	16 ± 39	27 ± 52	7.7 ± 28	0.419	0.430	0.084
Plasma IL-4 (pg/ml)	1.8 ± 2.8	2.4 ± 3.4	1.3 ± 2.3	0.387	0.844	0.234
Plasma IL-6 (pg/ml)	5.7 ± 5.5	41 ± 161	2.9 ± 3.7	0.013	**0.001**	**<0.001**
Plasma sIL-6R (pg/ml)	48 183 ± 14 538	52 941 ± 13 379	36 645 ± 10 573	0.055	**<0.001**	**<0.001**
Plasma IL-10 (pg/ml)	17 ± 16	15 ± 17	22 ± 81	0.858	0.015	0.026
Plasma TNF-α (pg/ml)	3.4 ± 9.5	12 ± 55	2.3 ± 9.1	0.289	0.103	**0.004**
Plasma TGF-β_2 (pg/ml)	5.1 ± 13	6.5 ± 17	1.3 ± 3.4	0.610	0.030	0.144
Plasma IFN-γ (pg/ml)	1260 ± 1872	1472 ± 2293	1810 ± 4388	0.804	0.866	0.902
Lymphocytes/μl	1635 ± 642	1861 ± 1232	2208 ± 868	0.788	**0.001**	0.019
% $CD3^+$	83 ± 8.6	83 ± 13	78 ± 6.7	0.568	**0.006**	**0.003**
$CD3^+/\mu l$	1362 ± 586	1560 ± 1099	1720 ± 638	0.851	**0.008**	0.046
% $CD3^+CD4^+$	50 ± 13	44 ± 13	46 ± 9.6	0.092	0.419	0.367
$CD3^+CD4^+/\mu l$	805 ± 387	771 ± 470	1040 ± 517	0.591	0.012	**0.009**
% $CD3^+CD8^+$	30 ± 12	35 ± 12	27 ± 6.7	0.094	0.275	**0.001**
$CD3^+CD8^+/\mu l$	509 ± 314	697 ± 579	573 ± 195	0.368	0.121	0.881
% DR^+	18 ± 8.0	19 ± 6.9	18 ± 5.5	0.624	0.903	0.388
$DR^+/\mu l$	299 ± 196	371 ± 327	403 ± 321	0.687	0.043	0.086
% $CD3^-CD16^+$	8.8 ± 6.7	9.8 ± 11	8.1 ± 5.2	0.696	0.912	0.554
$CD3^-CD16^+/\mu l$	138 ± 115	169 ± 197	168 ± 101	0.883	0.098	0.176
% $CD3^+25^+$	7.1 ± 3.9	5.7 ± 4.0	4.9 ± 2.2	0.095	**0.005**	0.935
$CD3^+25^+/\mu l$	115 ± 89	104 ± 100	108 ± 79	0.334	0.894	0.267

Table 2. continued

Parameter	Good graft function (Group 1) (n = 33)	Impaired graft function (Group 2) (n = 32)	Healthy controls (Group 3) (n = 63)	Group 1 vs. 2 *P*-value	Group 1 vs. 3 *P*-value	Group 2 vs. 3 *P*-value
% $CD19^+$	6.5 ± 3.6	7.5 ± 4.6	14 ± 4.5	0.410	**<0.001**	**<0.001**
$CD19^+$/μl	104 ± 74	122 ± 97	315 ± 238	0.601	**<0.001**	**<0.001**
% $CD3^+DR^+$	13 ± 10	16 ± 9.8	6.6 ± 4.6	0.221	**<0.001**	**<0.001**
$CD3^+DR^+$/μl	228 ± 210	343 ± 407	141 ± 139	0.485	0.074	0.011
% $CD3^+CD4^+DR^+$	4.7 ± 4.5	4.5 ± 3.5	2.6 ± 1.0	0.724	0.063	**0.004**
$CD3^+CD4^+DR^+$/μl	78 ± 84	94 ± 133	53 ± 24	0.788	0.795	0.807
% $CD3^+CD8^+DR^+$	7.7 ± 6.4	9.1 ± 6.8	2.9 ± 2.7	0.374	**<0.001**	**<0.001**
$CD3^+CD8^+DR^+$/μl	135 ± 135	202 ± 267	58 ± 49	0.537	**0.003**	**0.002**
% $lineage^-HLA\text{-}DR^+CD11c^+CD123^-$ DC1	4.1 ± 5.1	6.6 ± 11	7.0 ± 5.8	0.586	**0.002**	0.046
% $lineage^-HLA\text{-}DR^+CD11c^-CD123^+$ DC2	24 ± 18	27 ± 18	41 ± 26	0.502	**0.001**	**0.008**
DC1/DC2 ratio	0.42 ± 0.59	0.63 ± 1.3	1.1 ± 2.8	0.861	0.353	0.284

Adjustment for multiple testing was done according to the method of Bonferroni.
P-values of <0.01 were considered significant and are bold printed in the tables.

IL-3 (P = NS) as observed in patients with good graft function. We therefore speculated that the majority of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL in patients with impaired graft function might represent activated Th1 PBL. In order to substantiate this speculation, we investigated co-expression of these proteins in the immunosuppressive most recent 18 patients of the series of 65 patients.

Co-expression of Foxp3, IFN-γ, IL-4, and IL-10 by $CD4^+CD25^+$ PBL

Co-expression of Foxp3, IFN-γ, IL-4, and IL-10 by $CD4^+CD25^+$ PBL was studied in 11 patients with good- and seven patients with impaired graft function using four-color-fluorescence flow cytometry (Table 4). Nine patients with good- and six patients with impaired graft function had detectable $IFN\text{-}\gamma^+Foxp3^+$ $CD4^+CD25^+$ PBL (P = NS). Remarkably, six patients with good- but only one patient with impaired graft function showed more than 1.0% $IFN\text{-}\gamma^+Foxp3^+$ $CD4^+CD25^+$ PBL (P = 0.06) (Table 4).

In addition, patients with good graft function showed a trend towards higher frequencies of $IFN\text{-}\gamma^+IL\text{-}4^+$, $IFN\text{-}\gamma^+IL\text{-}10^+$, $IL\text{-}4^+Foxp3^+$, $IL\text{-}10^+Foxp3^+$, and $IL\text{-}4^+IL\text{-}10^+$ $CD4^+CD25^+$ PBL than patients with impaired graft function (Table 4). Conversely, none of the patients with good- but two patients with impaired graft function showed simultaneously undetectable $IFN\text{-}\gamma^+IL\text{-}4^+$, $IFN\text{-}\gamma^+IL\text{-}10^+$, $IL\text{-}4^+Foxp3^+$, $IL\text{-}10^+Foxp3^+$ and $IL\text{-}4^+IL\text{-}10^+$ $CD4^+CD25^+$ PBL (P = 0.060).

Figure 3 demonstrates the simultaneous expression of intracellular IFN-γ and intracellular Foxp3 in $CD4^+CD25^+$ T cells of a patient with a serum creatinine of 1.5 mg/dl, and the lack of expression of intracellular Foxp3 in $CD4^+CD25^+IFN\text{-}\gamma^+$ PBL of a patient with a serum creatinine of 2.5 mg/dl. The patient with good graft function had 2.9% $CD4^+CD25^+IFN\text{-}\gamma^+Foxp3^+$ PBL, the patient with impaired graft function 0%. Neopterin levels of the two patients were 17 and 32 nmol/l, respectively. The patient with good graft function was studied 2315 days post-transplant, the patient with impaired graft function 2969 days after transplantation. In addition, Fig. 3e and j demonstrate the three types of $CD4^+CD25^+$ PBL with IFN-γ- and/or Foxp3-expression that are detectable in the blood and that might have different immunoregulatory functions. The patient with good graft function showed $IFN\text{-}\gamma^+Foxp3^-$, $IFN\text{-}\gamma^+Foxp3^+$, and $IFN\text{-}\gamma^-Foxp3^+$ $CD4^+CD25^+$ PBL (Fig. 3e) whereas the patient with impaired graft function exhibited only $IFN\text{-}\gamma^-Foxp3^+$ but no $IFN\text{-}\gamma^+Foxp3^-$ and $IFN\text{-}\gamma^+Foxp3^+$ $CD4^+CD25^+$ PBL (Fig. 3j). $IFN\text{-}\gamma^+Foxp3^+$ and $IFN\text{-}\gamma^-Foxp3^+$ $CD4^+CD25^+$ PBL might represent immunosuppressive T cells whereas $IFN\text{-}\gamma^+Foxp3^-$ $CD4^+CD25^+$ PBL might represent immunostimulatory Th1 PBL. Evidently, patients with good graft function have $CD3^+CD4^+CD25^+Foxp3^+$ PBL that are supported in their immunosuppressive function by $CD3^+CD4^+CD25^+Foxp3^+IFN\text{-}\gamma^+$ PBL whereas patients with impaired graft function only have $CD3^+CD4^+$ $CD25^+Foxp3^+$ PBL and, as shown in the tables, $CD3^+$ $CD4^+CD25^+IFN\text{-}\gamma^+$ PBL that do not co-express Foxp3 and might represent immunostimulatory Th1 PBL.

$CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL and IFN-γ plasma levels

When frequencies of patients showing both detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL and detectable IFN-γ plasma levels were compared in the two patient groups, 22 patients with good (67%) but only 10 patients with impaired graft function (31%) showed both detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL as well as detectable IFN-γ

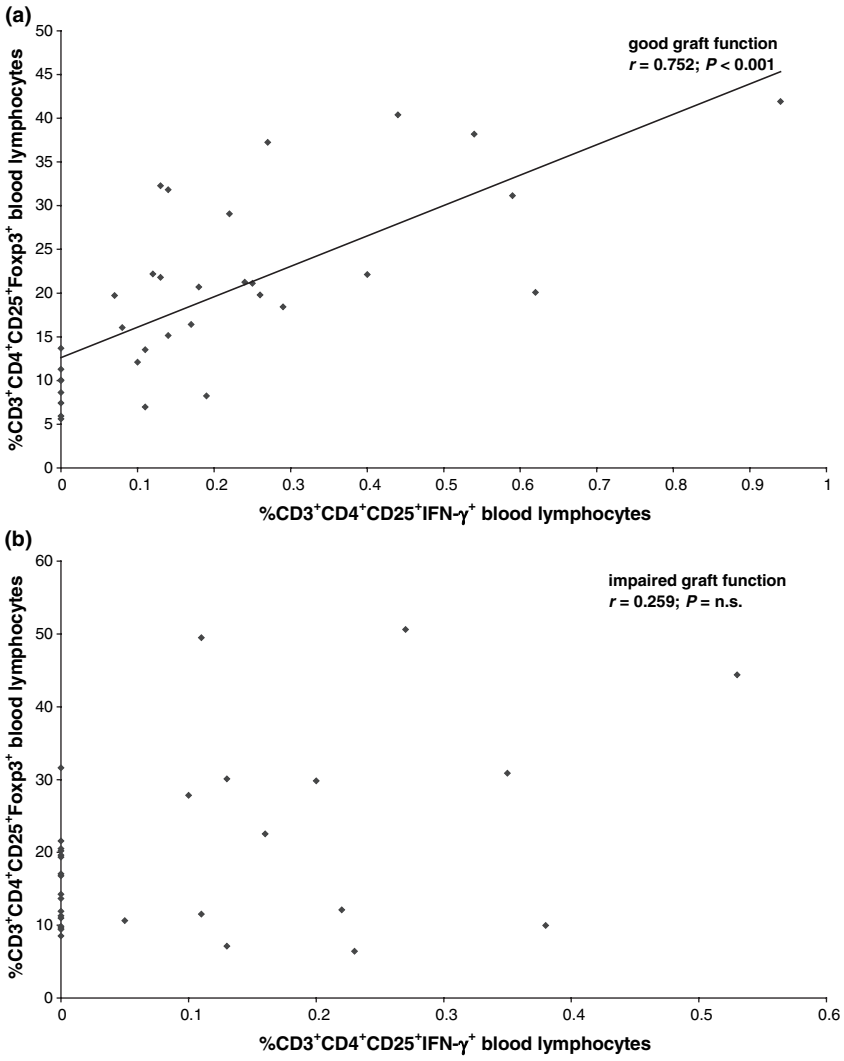

Figure 2 $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ and $CD3^+CD4^+CD25^+Foxp3^+$ PBL in 33 kidney transplant recipients with good graft function (creatinine ≤ 1.8 mg/dl) and 32 transplant recipients with impaired graft function (creatinine ≥ 2.0 mg/dl). Whereas high $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL were associated with high $CD3^+CD4^+CD25^+Foxp3^+$ PBL in patients with good graft function (r = 0.752; P < 0.001) (a), there was no corresponding association in patients with impaired graft function (r = 0.259; P = 0.159) (b). Patients with good graft function and high $CD3^+CD4^+CD25^+Foxp3^+$ PBL showed high $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL, whereas among patients with impaired graft function especially those individuals with low $CD3^+CD4^+CD25^+Foxp3^+$ PBL had high $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL.

plasma levels (P = 0.004) (Fig. 4). Noticeably, there were only two patients with good- but four patients with impaired graft function showing detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL in the absence of a measurable IFN-γ plasma level. The data suggest that detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL and detectable IFN-γ plasma levels are more frequently present in kidney transplant with good graft function rather than in patients with impaired graft function, and that $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL in patients with good graft function might affect IFN-γ plasma levels. Conversely, 16 patients with impaired graft function (50%) but only seven patients with good graft function (21%) had measurable IFN-γ plasma levels in the absence of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL (P = 0.015) (Fig. 4), suggesting that in the patients with impaired graft function, plasma IFN-γ might be up-regulated independently of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL.

$CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL, rejection, and infection

Four patients with good graft outcome and four patients with impaired graft function had a history of acute rejection post-transplant, whereas four patients in each group had a history of acute infection after transplantation (Table 1). The four patients with good graft outcome and prior rejection had 0%, 0.11%, 0.40%, and 0.54% $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL; the four patients with impaired graft function and a history of acute rejection had 0%, 0%, 0.13%, and 0.20% $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL. The four patients with good graft outcome and prior infections had 0%, 0%, 0%, and 0.59% $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL, the four patients with impaired graft outcome and prior acute infection had 0%, 0%, 0%, and 0.13% $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL. The data show (i) that patients with good graft function and patients with impaired graft function had similar

Table 3. Associations of CD3+CD4+CD25+IFN-γ+ PBL and CD3+CD4+CD25+Foxp3+ PBL with CD3+CD4+CD25+ and CD3+CD8+CD28− PBL subsets, lymphocyte subpopulations, and plasma cytokines in transplant recipients with good (crea ≤ 1.8 mg/dl) or impaired (crea ≥ 2.0 mg/dl) graft function*.

	Good graft function (*n* = 33)				Impaired graft function (*n* = 32)			
	%CD3+CD4+ CD25+IFN-γ+		%CD3+CD4+ CD25+Foxp3+		%CD3+CD4+ CD25+IFN-γ+		%CD3+CD4+ CD25+Foxp3+	
Parameter	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value
% CD3+CD4+CD25+IFN-γ+	–	**–**	0.752	**<0.001**	–	–	0.291	0.106
CD3+CD4+CD25+IFN-γ+/μl	0.888	**<0.001**	0.632	**<0.001**	0.920	**<0.001**	0.081	0.666
% CD3+CD4+CD25+Foxp3+	0.752	**<0.001**	–	–	0.259	0.159	–	–
CD3+CD4+CD25+Foxp3+/μl	0.451	0.010	0.734	**<0.001**	0.237	0.192	0.404	0.024
% CD3+CD4+CD25+IL-2+	0.581	**<0.001**	0.656	**<0.001**	0.368	0.038	0.340	0.061
CD3+CD4+CD25+IL-2+/μl	0.456	**0.009**	0.626	**<0.001**	0.404	0.022	0.019	0.920
% CD3+CD4+CD25+IL-4+	0.618	**<0.001**	0.528	**0.002**	0.440	0.012	0.300	0.101
CD3+CD4+CD25+IL-4+/μl	0.529	**0.002**	0.452	**0.009**	0.439	0.012	0.129	0.489
% CD3+CD4+CD25+IL-10+	0.642	**<0.001**	0.701	**<0.001**	0.197	0.279	0.458	0.010
CD3+CD4+CD25+IL-10+/μl	0.560	**0.001**	0.622	**<0.001**	0.179	0.327	0.445	0.012
% CD3+CD4+CD25+IL-12+	0.634	**<0.001**	0.574	**<0.001**	0.394	0.026	0.483	**0.006**
CD3+CD4+CD25+IL-12+/μl	0.528	**0.002**	0.487	**0.005**	0.364	0.040	0.273	0.137
CD3+CD8+CD28−Foxp3+/μl	−0.521	**0.002**	−0.379	0.033	−0.228	0.209	−0.428	0.016
% CD3+CD4+CD127+	0.494	**0.003**	0.419	0.015	0.136	0.459	0.196	0.290
CD3+CD8+CD127+/μl	−0.468	**0.007**	−0.407	0.021	0.031	0.867	−0.212	0.260
Lymphocytes/μl	−0.467	**0.007**	−0.365	0.040	0.030	0.869	−0.286	0.119
CD3+/μl	−0.482	**0.005**	−0.379	0.032	0.041	0.824	−0.357	0.049
CD3+CD8+/μl	−0.542	**0.001**	−0.336	0.060	−0.136	0.458	−0.299	0.102
% DR+	−0.528	**0.002**	−0.544	**0.001**	−0.555	**0.001**	−0.096	0.608
DR+/μl	−0.613	**<0.001**	−0.550	**0.001**	−0.260	0.151	−0.321	0.079
% CD3+25+	0.098	0.593	0.712	**<0.001**	0.361	0.043	0.644	**<0.001**
CD19+/μl	−0.489	**0.005**	−0.427	0.015	−0.114	0.535	−0.392	0.029
% CD3+DR+	−0.446	0.011	−0.485	**0.005**	−0.436	0.013	−0.132	0.478
CD3+DR+/μl	−0.592	**<0.001**	−0.538	**0.002**	−0.309	0.085	−0.305	0.095
CD3+CD4+DR+/μl	−0.519	**0.002**	−0.546	**0.001**	−0.234	0.198	−0.378	0.036
CD3+CD8+DR+/μl	−0.487	**0.005**	−0.360	0.043	−0.207	0.257	−0.281	0.125
% lineage−HLA-DR+CD11c+CD123− DC1	−0.472	**0.006**	−0.448	0.010	−0.205	0.260	−0.300	0.101
Plasma IL-3 (pg/ml)	−0.548	**0.001**	−0.359	0.043	0.085	0.648	−0.064	0.736
Plasma IFN-γ (pg/ml)	0.313	0.081	0.184	0.312	−0.275	0.134	0.024	0.899

Adjustment for multiple testing was done according to the method of Bonferroni.

P-values of <0.01 were considered significant and are bold printed in the tables.

*Associations of %CD3+CD4+CD25+IFN-γ+ PBL and %CD3+CD4+CD25+Foxp3+ PBL with all parameters listed in Table 2 were calculated using the Spearman rank correlation test .

Only parameters showing a *P*-value of <0.01 with %CD3+CD4+CD25+IFN-γ+ PBL and/or % CD3+CD4+CD25+Foxp3+ PBL in patients with good graft function are listed.

frequencies of previous acute rejections or infections, and (ii) that patients with prior acute rejection or prior acute infection had similar frequencies of detectable CD3+CD4+CD25+IFN-γ+ PBL.

CD3+CD4+CD25+IFN-γ+ PBL, graft status in terms of days post-transplant, and chronic allograft nephropathy

Four patients suffered from biopsy-proven chronic allograft nephropathy and all four patients had undetectable CD3+CD4+CD25+IFN-γ+ PBL. In this context, it might be interesting that of the patients with more than 1000 days post-transplant follow-up, 13 of 17 (76%) with good graft outcome had detectable CD3+CD4+CD25+ IFN-γ+ PBL, in contrast to only nine of 20 (45%) with impaired graft function ($P = 0.05$), suggesting that an absence of CD3+CD4+CD25+IFN-γ+ PBL might support the development of chronic allograft nephropathy.

Discussion

Patients with acute rejection have been reported to possess lower DC2, lower DC1, lower Treg and/or lower Ts lymphocytes than patients with an uncomplicated

Table 4. Co-expression of Foxp3, IFN-γ, IL-4, and IL-10 in CD4$^+$CD25$^+$ PBL of transplant recipients with good (crea ≤ 1.8 mg/dl) or impaired (crea ≥ 2.0 mg/dl) graft function.

		% of CD4$^+$CD25$^+$ PBL double-positive for					
Patient	Crea (mg/dl)	INF-γ^+ Foxp3$^+$	INF-γ^+ IL-4$^+$	INF-γ^+ IL-10$^+$	IL-4$^+$ Foxp3$^+$	IL-10$^+$ Foxp3$^+$	IL-4$^+$ IL-10$^+$
A	1.1	0.1	0.25	0	0.29	0	0.44
B	1.1	0	0.05	0.05	0.17	0	0
C	1.3	4.04	0.63	1.88	1.16	2.42	1.66
D	1.3	5.12	2.53	2.09	1.16	1.15	4.83
E	1.3	0.08	0	0	0.03	0.07	0.04
F	1.3	0	0	0.09	0.56	0	0.12
G	1.4	1.51	2.05	0.51	1.91	0.61	2.19
H	1.4	31.3	40.14	15.89	19.28	7.8	2.77
I	1.6	1.36	0.27	0.37	0.3	0	0
J	1.7	1.13	0	1	0	0.17	2.63
K	1.7	0.17	0.24	0	0	0.58	0.92
L	2.3	1.12	0	0	0.28	0.37	0
M	2.3	0.17	0	0.12	0.14	0	0
N	2.6	0.05	0	0	0	0	0
O	2.8	0.38	0.12	0.03	0.22	0.88	5.52
P	3.4	0.21	0.03	0	0.03	0.04	0
Q	4.2	0.29	0.21	0.08	0.39	0	0.08
R	4.6	0	0	0	0	0	0

postoperative course [1,10–14]. Because immunosuppressive drugs have the potential to induce DC2, Treg, and Ts *in vitro*, it was suggested by some authors that early post-transplant high dose immunosuppression may induce immunosuppressive DC2, Treg, and Ts, and that these regulatory cells might keep the allo-response down-regulated when doses of immunosuppressive drugs are reduced during post-transplant follow up [1,15–18]. In contrast, other authors suggested that Treg generation post-transplant may be prevented by strong immunosuppression [12]. Others reported higher Treg numbers in kidney transplant recipients on sirolimus than that in recipients on cyclosporine [19,20]. To minimize the influence of very high immunosuppressive drug doses on Treg and IFN-γ plasma levels in our study, we investigated patients who were at least 100 days post-transplant and we avoided comparing extremes of patients with perfect or strongly impaired graft function. Instead, we chose 65 consecutive transplant outpatients and separated them into two groups, with serum creatinine values of ≤1.8 or ≥2 mg/dl. None of the patients had a current acute rejection or infection. Five patients with a serum creatinine of ≥2 mg/dl show evidence of chronic graft dysfunction. As shown in Table 1 and in the Result section, there was no trend of stronger immunosuppression in patients with impaired graft function and there was no evidence that late post-transplant drug doses were associated with low proportions of CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ and CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ PBL, or low IFN-γ plasma levels. Moreover, there was no evidence that induction treatment with antibodies or the occurrence of previous acute rejections or infections accounted for the less frequent occurrence of CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL in patients with impaired graft function, when compared to patients with good graft function. CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL was the only cell subset that was higher in patients with good graft function than that in patients with impaired graft function. The data shown in Table 2 illustrate that immunosuppressive drugs have a general effect on cell subset levels in transplant recipients. However, the similar immunosuppressive drug doses in patients with good and impaired graft function appear to affect these cell subsets similarly in the two patient groups.

Our data support the hypothesis that patients with good graft function have CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL that co-express Foxp3, suggesting that these cells are immunoregulatory T cells. In contrast, CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL of patients with impaired graft function lack intracellular Foxp3 co-expression, suggesting that these PBL are not immunoregulatory and represent activated Th1 PBL that maintain an immune response against the allograft. The immunoregulatory role of CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL in patients with good graft outcome is substantiated by the finding that CD3$^+$CD4$^+$CD25$^+$IFN-γ^+ PBL and CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ PBL showed similar associations with low CD19$^+$ B cells, low activated CD3$^+$CD4$^+$DR$^+$ PBL, low DC1, low CD8$^+$ PBL expressing the phenotype of activated cytotoxic T PBL (CD3$^+$CD8$^+$DR$^+$) and/or suppressor T PBL (CD3$^+$CD8$^+$CD28$^-$Foxp3$^+$), and low plasma IL-3. We

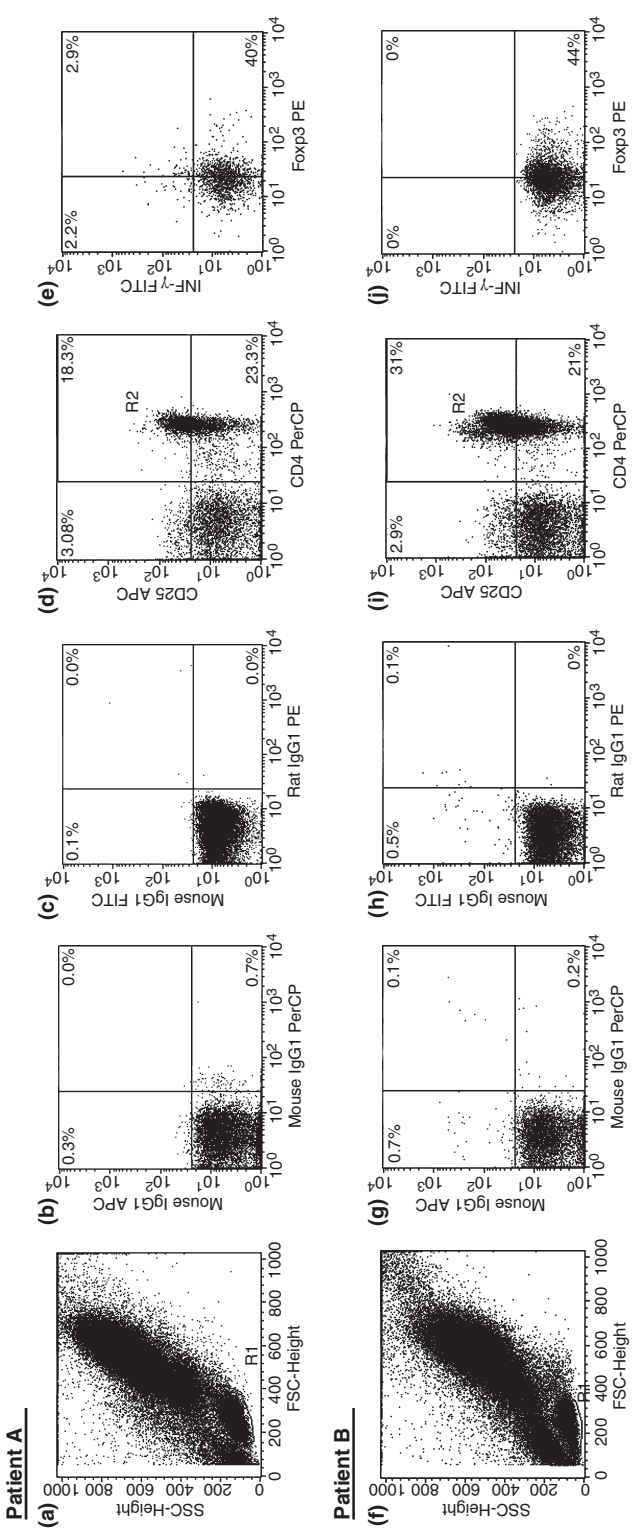

Figure 3 Simultaneous expression of intracellular IFN-γ and Foxp3 in $CD3^+CD4^+CD25^+$ T cells. Patient A had a serum creatinine of 1.5 mg/dl, patient B a serum creatinine of 2.5 mg/dl. Patient A showed a co-expression of intracellular IFN-γ and Foxp3 in $CD3^+CD4^+CD25^+$ T cells (2.9% $CD4^+CD25^+IFN\text{-}\gamma^+Foxp3^+$ PBL; e) whereas patient B showed no co-expression of intracellular IFN-γ and Foxp3 in $CD3^+CD4^+CD25^+$ T cells (0% $CD4^+CD25^+IFN\text{-}\gamma^+Foxp3^+$ PBL; j). (a–d and f–i) Gating strategy: gating firstly lymphocytes in a forward/side scatter gate, thereafter appropriate isotype controls, then $CD4^+CD25^+$ PBL, and lastly intracellular expression of Foxp3 and cytokines.

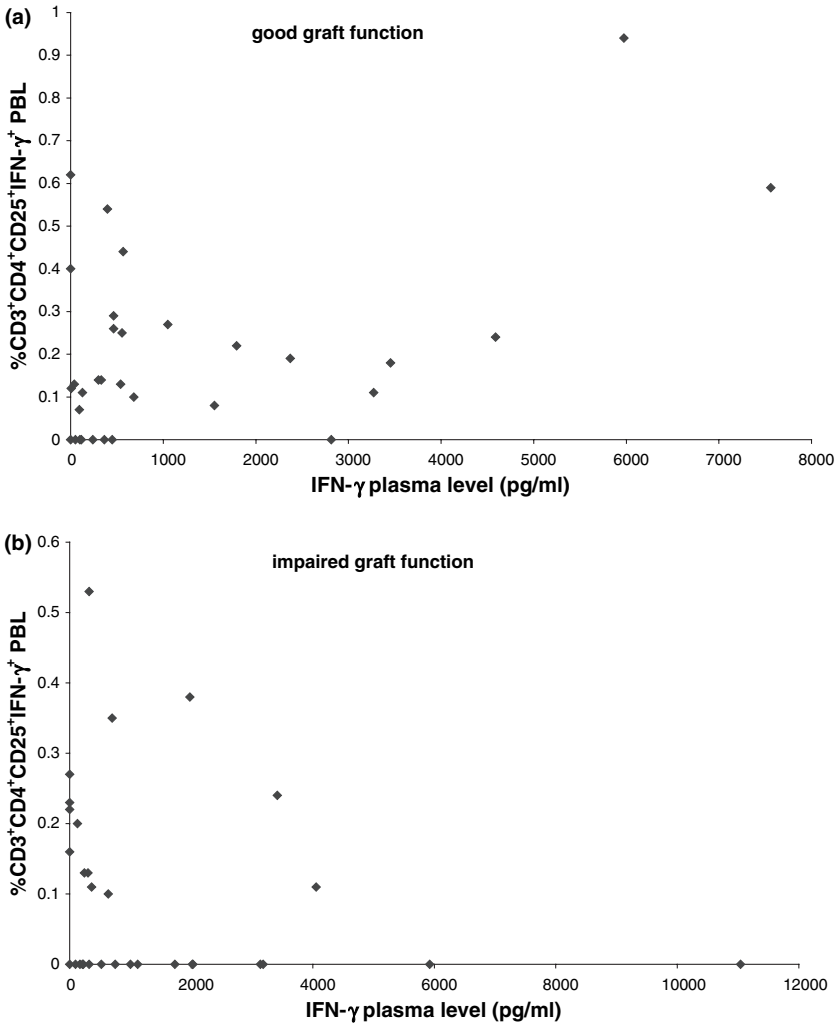

Figure 4 $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL and IFN-γ plasma levels in 33 kidney transplant recipients with good graft function (creatinine ≤ 1.8 mg/dl) and 32 transplant recipients with impaired graft function (creatinine ≥ 2.0 mg/dl). Whereas 22 patients with good graft function (67%) had detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL as well as detectable IFN-γ plasma levels (a), only 10 patients with impaired graft function (31%) showed measurable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL and measurable plasma IFN-γ ($P = 0.004$) (b). Two patients with good graft function and four patients with impaired graft function showed detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL but undetectable IFN-γ plasma levels. The data suggest that detectable $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL and detectable IFN-γ plasma levels are more frequently present in kidney transplant with good graft function than in patients with impaired graft function, and that $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL in patients with good graft function might affect IFN-γ plasma levels. Conversely, 16 patients with impaired graft function (50%) but only seven patients with good graft function (21%) had measurable IFN-γ plasma levels in the absence of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL ($P = 0.015$), suggesting that in patients with impaired graft function plasma IFN-γ might be up-regulated independently of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL.

speculate that $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL in patients with good graft function down-regulate $CD19^+$ B cells, immunostimulatory DC1, activated $CD4^+$ and $CD8^+$ T cells as well as plasma IL-3 levels.

There is evidence of strong co-expression of Foxp3, IFN-γ, IL-4, and IL-10 by $CD4^+CD25^+$ PBL of patients with good graft function, and a sporadic and lower co-expression of some of these proteins by $CD4^+CD25^+$ PBL of patients with impaired graft function. Our data suggest a higher frequency of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+Foxp3^+$ PBL in patients with good graft function, and that some of the $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+Foxp3^+$ PBL in patients with good graft function produce IL-4 and IL-10. IL-4 and IL-10 secretion of these T lymphocytes might amplify the immunosuppressive effect of these cells on the immune system. Patients with good graft function

appear to have $CD3^+CD4^+CD25^+Foxp3^+$ PBL that are supported in their immunosuppressive function by $CD3^+CD4^+CD25^+Foxp3^+IFN\text{-}\gamma^+$ and $CD3^+CD4^+CD25^+Foxp3^+IFN\text{-}\gamma^+IL\text{-}4^+IL\text{-}10^+$ PBL, and presumably also by classical Treg and Th2 lymphocytes expressing the phenotype $CD3^+CD4^+CD25^+Foxp3^+IL\text{-}4^+IL\text{-}10^+$ and $CD3^+CD4^+CD25^+IL\text{-}4^+IL\text{-}10^+$, respectively [4,21]. In contrast, patients with impaired graft function only have $CD3^+CD4^+CD25^+Foxp3^+$ PBL and $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL that do not co-express Foxp3 and might represent immunostimulatory Th1 PBL. Treg-producing IL-10 but not IFN-γ were shown to inhibit Th1 and cellular cytotoxic immune responses *in vitro* and *in vivo* [4,22]. Treg are not a homogeneous cell population but rather are composed of different cell subsets which have in common the suppression of cellular and humoral immune responses [23–27]. Additional subsets of suppressive Treg were described which produce TGF-ß and express CD62, cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR), and/or lymphocyte activation gene 3 (LAG-3) [23–27].

Our data provide support for the hypothesis that $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL might contribute in any one of the different forms to the high IFN-γ plasma levels observed in patients with good graft outcome and that the allograft acceptance might be mediated in part by high-IFN-γ plasma levels. IFN-γ might in part originate from $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL. Conversely, our data show that IFN-γ plasma levels in patients with impaired graft function originate from other cells than $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL, presumably NK cells or $CD8^+$ T PBL or $CD3^+CD8^+Foxp3^+$ regulatory $CD8^+$ T cells [28,29]. Activated cytotoxic effector cells might mediate the immune response against the allograft that is indicated by elevated creatinine and neopterin in these patients.

Interestingly, patients with good graft function showed an association of high $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL with low plasma IL-3 levels. IL-3 was shown to amplify T-cell proliferation [30]. Low IL-3 plasma levels might inhibit proliferation of activated graft-reactive T cells.

The statistical associations found in this study might be a reflection of complex interactions of immune mechanisms that take place *in vivo* and result in the maintenance of graft acceptance. An immunosuppressive capacity of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ Treg in mice was described by Sawitzki *et al.* [7] and Wood and Sawitzki [8]. As proposed by Wood and Sawitzki, IFN-γ might indirectly prevent further T-cell activation by creating a microenvironment that influences the function of antigen-presenting cells as a result of IFN-γ-induced nitric oxide synthase, indoleamine-2,3-dioxygenase and heme oxygenase-1 expression [8]. Because patients with impaired graft function showed lower absolute numbers of $CD3^+CD4^+CD25^+IFN\text{-}\gamma^+$ PBL than patients with good long-term graft function or healthy controls (Table 2), a reduction of this immunoregulatory-cell subset might contribute to graft damage. Our data are in line with reports of others describing abnormally low Treg numbers in patients with rejections [31–33]. Abnormally decreased Treg numbers were observed in renal transplant recipients with chronic rejection, whereas Treg numbers in patients with graft acceptance were similar to those of healthy volunteers [31]. Patients with chronic graft-versus-host disease (cGVHD) showed a reduced frequency of $CD3^+CD4^+CD25^+Foxp3^+$ Treg as compared to patients without cGVHD or healthy controls [10], and Treg depletion via treatment with anti-CD25 monoclonal antibody in mice significantly enhances $CD8^+$ responses to influenza A virus [32], or rejection of skin and heart allografts [33]. In this context it might be of interest that donor-specific hyporesponsiveness after renal transplantation was found to be associated with Treg recruitment in the graft [34].

Our data provide evidence for the existence of a patient subgroup with good graft function despite abnormally high IFN-γ plasma levels (Fig. 4). These patients show high levels of $CD3^+CD4^+CD25^+$ PBL expressing IFN-γ and Foxp3. We speculate that $CD3^+CD4^+CD25^+Foxp3^+IFN\text{-}\gamma^+$ PBL may contribute to the maintenance of immunological quiescence and that the absence of $CD3^+CD4^+CD25^+Foxp3^+IFN\text{-}\gamma^+$ PBL promotes chronic graft dysfunction.

Authorship

VD, CN, MS and GO designed and performed the research/study, analyzed the data and wrote the manuscript. RW, FR and SY treated the patients and analyzed the clinical data.

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