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CD40L, CD28, and CTLA-4 expression on CD4+ T cells in kidney graft recipients: A relationship with post-transplantation clinical course

Agata Kosmaczewska *[,1], Maria Magott-Procelewska [1], Irena Frydecka, Lidia Ciszak, Dorota Bocko, Aleksandra Szteblich, Piotr Kusnierczyk, Dariusz Patrzalek, Piotr Szyber, Marian Klinger

Department of Experimental Therapy, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wroclaw, Poland
Department of Nephrology and Transplant Medicine, Medical University of Wroclaw, Traugutta 57/59, 50-417 Wroclaw, Poland
Department of Hematology, Medical University of Wroclaw, Pasteura 4, 50-367 Wroclaw, Poland
Department of Clinical Immunology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wroclaw, Poland
Department of Vascular, General, and Transplantation Surgery, Medical University of Wroclaw, Poniatowskiego 2, 50-325 Wroclaw, Poland

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Abstract

Background: Experimental studies have demonstrated that the intensity of alloreactivity against a transplanted organ results from an interaction of positive (CD40/CD40L and B.7/CD28) and inhibitory (B.7/CTLA-4) signals between antigen-presenting cells (APCs) and T lymphocytes.
Methods: We examined the CD40L, CD28, and both surface (s) and intracellular (i) CTLA-4 expressions on freshly drawn and anti-CD3+rIL-2-stimulated peripheral blood CD4+ T cells in groups of kidney transplant recipients in relation to distinct clinical course using the tri-color immunofluorescence method.
Results: The median proportions of freshly isolated CD3+/CD4+/CTLA-4+ and CD3+/CD4+/CD40L+ cells in all groups of graft recipients were higher than in control subjects. In patients with stable graft function (SGF), non-significantly higher sCTLA-4, significantly higher iCTLA-4 expression, and significantly lower CD40L expression on freshly drawn CD4+ T cells compared with recipients with chronic allograft nephropathy (CAN) were found. Moreover, CD4+ T cells from SGF patients showed a higher potential to express sCTLA-4 and CD40L molecules and to down-regulate the CD28 molecule in response to ex vivo stimulation than those from patients with CAN. In patients without acute graft rejection (NAGR), a markedly higher proportion of freshly drawn CD3+/CD4+/iCTLA-4+ cells compared with patients with acute graft rejection (AGR) and an up-regulation of the median percentage of CD3+/CD4+/CD40L+ cells after ex vivo stimulation was found.
Conclusions: In patients with SGF, peripheral blood CD4+ T cells exhibited a higher potential to express surface CTLA-4 and CD40L and to down-regulate CD28 costimulatory molecules in response to ex vivo stimulation, indicating a relationship between the expression patterns of both costimulatory and inhibitory molecules in CD4+ T cells and clinical course after renal transplantation.

Keywords: Costimulatory molecules; CD40L (CD154); CD28; CTLA-4 (CD152); Kidney transplantation; Clinical course

1. Introduction

Experimental data have shown that both transplant organ rejection and tolerance are active processes involving both costimulatory and inhibitory pathways. One pathway that has

* Corresponding author. Department of Experimental Therapy, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wroclaw, Poland. Tel.: +48 71 337 11 72; fax: +48 71 337 13 82.
E-mail address: kosmacz@immuno.iitd.pan.wroc.pl (A. Kosmaczewska).
[1] These authors contributed equally to this work.

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received significant attention in transplantation and which is crucial in T-cell activation is mediated by CD40L (CD154). This molecule, a member of the TNF family, is transiently expressed on activated T cells, primarily CD4+ T cells [1–3]. The expression of CD40L represents one of the earliest and most specific T-lymphocyte activation markers during immune reaction. Experimental data showed that the interaction of CD40L with CD40 on antigen-presenting cells (APCs) directly transduces a positive signal to the cell's interior. It has also been found that part of the mechanism of action of the CD40/CD40L pathway in supporting T-cell immune responses is indirect, i.e. through the induction of CD28 ligands [B.7: B.7-1 (CD80) and/or B.7-2 (CD86)] and the resultant activation of the CD28 pathway.

CD28 is the best understood T-cell costimulatory molecule and is expressed constitutively on almost all CD4+ T cells and on about 50% of CD8+ T cells. Upon ligation of CD28 with its B.7 ligands, CD28 transduces a signal that enhances T-cell proliferation and cytokine secretion (Il-2, IFN-gamma, and TNF) and stabilizes the cytokine gene transcripts [4]. Without CD28 costimulation, T-cell responses are quantitatively weaker and are only seen transiently at high antigen concentrations [5,6].

Costimulatory amplification loops between APCs and helper T cells are ultimately modulated by the subsequent expression of negative signals from CTLA-4 (cytotoxic T lymphocyte-associated antigen-4; CD152), only minimally expressed on resting cells and transiently up-regulated on activated T cells after 48–72 h of stimulation [7]. The expression of CTLA-4 within the T-cell population is restricted to the subset of T cells that also express CD28 on the cell surface [8]. CTLA-4 mRNA and protein synthesis is induced by the ligation of CD28 with its B.7 ligands [8]. However, it is important to note that CTLA-4 binds the same B.7 ligands as does CD28, but has an affinity 10–50 times higher than that of CD28. CTLA-4 inhibits the induction of the IL-2 receptor alpha chain (CD25), CD69 molecule expression, CD3/CD28-induced IL-2 mRNA accumulation, and the secretion of IL-2 [9,10]. CTLA-4 ligation also augments the production of immunosuppressive TGF-beta [11].

The outcome of immune response results from a balance between positive and negative regulatory signals. Recently, experimental transplant studies demonstrated that the interactions of CD40L with CD40 and CD28/CTLA-4 with their specific B.7 ligands have decisive roles in antigenic and allogenic responses and, in consequence, in the acceptance or rejection of a renal allograft [12,13]. However, studies on the expressions of these molecules on peripheral blood lymphocytes (PBLs) in human renal graft recipients are still very limited. Shoker et al. [14] found higher CD40L gene expression in the peripheral CD4+ T cells of patients with acute graft rejection (AGR) and chronic allograft nephropathy (CAN) than in recipients with stable graft function (SGF) and normal subjects. After ex vivo stimulation with PWM, CD40L gene expression increased in all studied subjects. Recently, Lederer et al. [15] showed that patients with excellent kidney graft function early after transplantation displayed significantly reduced expression of CD40L surface molecules on CD4+ cells after PMA+ionomycin stimulation. Recipients with a chronic dysfunction of the renal graft showed markedly more CD4+ cells expressing CD40L compared with the other transplanted groups [15]. In turn, Kato et al. [16] observed an increased number of PB CD28−/CD4+ T cells in patients who had long graft survival of more than 7 years. All these studies strongly support the notion that costimulatory molecules may contribute to tolerance and/or rejection of a kidney allograft. No studies on the expression of the down-regulatory CTLA-4 molecule on PB T cells in different groups of graft recipients have been reported so far according to our knowledge.

2. Objective

Our study was designed to systematically analyze CD40L, CD28, and both surface (s) and intracellular (i) CTLA-4 expressions on freshly drawn and anti-CD3+rIL-2-stimulated PB CD4+ T cells from kidney transplant recipients with the aim to estimate possible differences related to the varied post-transplantation course.

3. Material and methods

3.1. Patients

Eighty recipients of kidney allografts (35 females and 45 males), between 18 and 67 (mean 43) years of age, who underwent a transplantation between January 1998 and March 2003, were studied after giving informed consent. The cause of end-stage renal disease was chronic glomerulonephritis in 39 patients (48.7%), interstitial nephritis in 9 (11.3%), polycystic kidney disease in 7 (8.7%), diabetic nephropathy in 5 (6.3%), hypertensive nephropathy in 3 (3.7%), and other (or unknown) etiologies in 17 (21.3%). All individuals received calcineurin inhibitor (62 patients received cyclosporin A and 18 patients received tacrolimus), azathioprine or mycophenolate mofetil, and steroids. In order to exclude inflammatory reactions, the serum levels of C-reactive protein (CRP) were measured using a highly sensitive assay. Patients with values higher than 0.5 mg/dl were excluded from the study. No evidence of bacterial or viral infections was observed when blood samples were taken.

The patients were classified into four groups based on clinical course and histopathological evaluation: acute graft rejection (AGR, $n=17$), confirmed by a renal graft biopsy and categorized according to the Banff criteria, with all rejection episodes corticosteroid sensitive; non-acute graft rejection (NAGR, $n=29$), including patients exhibiting an uneventful course in the first three post-transplantation months, reaching a serum creatinine concentration below 1.5 mg/dl; chronic allograft nephropathy (CAN, $n=10$), with deteriorating graft function, appearing after 1 year post-transplantation, with biopsy-proven features of chronic rejection; and stable graft function (SGF, $n=24$), including patients presenting an uneventful course without acute rejection episodes, preserving normal renal graft function in long-term follow-up (with serum creatinine levels below 1.5 mg/dl after an observation period of at least 18 months). There were no statistically significant differences between the four studied groups in demographic and clinical characteristics including mean age, gender, cause of ESRD, HLA mismatches, and mean trough levels of cyclosporine A or tacrolimus (Table 1).

The studies were performed during the first 3 months in the AGR and NAGR groups and between 18 and 42 months after renal transplantation in patients with SGF and CAN. The control samples consisted of peripheral blood mononuclear cells from 27 healthy volunteers (C).

3.2. Isolation of cells and culture conditions

Peripheral blood mononuclear cells (PBMCs) were separated by buoyant density-gradient centrifugation on Lymphoflot (Biotest, Germany) from freshly drawn peripheral venous blood (PB) and washed three times in

Table 1
Demographic characteristic of renal transplant recipients

	AGR (n=17)	NAGR (n=29)	SGF (n=24)	CAN (n=10)	All (%) (n=80)
Gender					
Female	6	14	11	4	35 (43.7%)
Male	11	15	13	6	45 (56.3%)
Mean age (years)	44.8±10.7	47.6±7.6	39.4±9.8	41±9.9	
Mean (S.D.) number of HLA mismatches	3.1±0.8	3.3±0.7	3.2±0.6	3.4±0.7	3.2±0.7
Cause of ESRD					
Glomerulonephritis	8	11	14	6	39 (48.7%)
Polycystic kidney disease	1	5	1	0	7 (8.7%)
Interstitial nephritis	1	4	3	1	9 (11.3%)
Hypertensive nephropathy	2	1	0	0	3 (3.7%)
Diabetic nephropathy	2	2	1	0	5 (6.3%)
Other or unknown	3	6	5	3	17 (21.3%)
Immunosuppressive medications					
Cyclosporine A, mean trough level (ng/ml)	n=11, 217±145	n=24, 208±102	n=21, 168±52	n=6, 166±30	
Tacrolimus, mean trough level (ng/ml)	n=6, 11.4±4.4	n=5, 10.9±4.6	n=3, 8.6±2.7	n=4, 12.1±3.6	
Mycophenolate mofetil, mean dose (g/day)	n=10, 1.8±0.4	n=15, 1.8±0.2	n=7, 2.0	n=6, 1.2±0.7	
Azathioprine, mean dose (mg/day)	n=7, 120±21	n=14, 122±34	n=17, 73±19	n=4, 75±10	

0.9% saline. The PBMCs were resuspended at 1×10^6 PBMC/ml in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Flow Laboratories, UK), L-glutamine, and 50 mg/ml gentamycin (Gibco), then cultured with 10 ng/ml of anti-CD3 monoclonal antibody (mAb) (Ortho, Neckargemund, Germany) and 500 U/ml of rIL-2 (Eurocetus, Amsterdam, The Netherlands). Control cultures without stimulants were included in each experiment. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 72 h. In our experimental model, rIL-2 was used as a second signal to obtain optimal stimulation of T cells [17].

3.3. Flow cytometric analysis

All experiments on fresh and cultured cells were carried out by triple labeling with PerCP-, FITC-, or RPE-conjugated monoclonal antibodies (MoAbs). The MoAbs used in all experiments were as follows: anti-CD3/PerCP (Becton Dickinson, San Jose, CA, USA), anti-CD4/RPE and anti-CD4/FITC (Becton Dickinson, San Jose, CA, USA), pure anti-CTLA-4 and anti-CTLA-4/RPE (PharMingen, San Diego, CA, USA), anti-CD40L/RPE (Becton Dickinson, San Jose, CA, USA), and anti-CD28/FITC (Serotec, UK).

Since the analysis of the expressions of CD40L, CD28, and CTLA-4 molecule within the CD4+ population would be complicated by the fact that CD4 antigen is also present on monocytes, we performed our studies using a triple immunostaining method (a T-cell marker, a T-cell subset (CD4) marker, and a CD40L, CD28, sCTLA-4, or iCTLA-4 marker). Briefly, after isolation the cells were washed twice in phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) containing 0.5% Tween-20. The cells were then incubated for 30 min with the antibodies described above and excess, unbound antibodies were removed by two washes with PBS. Following these washes, the cells were fixed with 1.5% paraformaldehyde in PBS and analyzed by flow cytometry using a FACScalibur flow cytometer (Becton Dickinson). The estimated detection limit of the FACScalibur flow cytometer is 750 molecules of equivalent soluble fluorescein. For intracellular detection of CTLA-4, the cells were first incubated for 30 min at 4 °C in the dark with pure anti-CTLA-4 MoAb to block surface CTLA-4 molecules, then fixed and permeabilized according to the method previously described [18,19], and then stained with CD3/PerCP, CD4/FITC, and CTLA-4/RPE antibodies. Negative controls were always done by omitting the MoAb and by incubating the cells with mouse Ig of the same isotype as the MoAbs conjugated with PerCP, FITC, or RPE. The results were expressed as the proportion of CD3+/CD4+ cells expressing CTLA-4 (on the cell surface and intracellularly), CD40L, or CD28 molecules. At least 10,000 events per sample were analyzed.

3.4. Statistical analysis

The results of the expressions of CD28, CD40L, and CTLA-4 from all the studied groups were compared by the Kruskal–Wallis test. The Wilcoxon's test was used for dependent samples. The results were expressed as the median and lower and upper quartiles. The level of statistical significance was set at 0.05.

4. Results

4.1. Expression of CD40L on freshly isolated and anti-CD3+rIL-2-stimulated CD4+ T cells in kidney transplant recipients and healthy controls

The frequency of CD40L+/CD4+ T cells in freshly drawn PB was significantly higher in recipients with AGR, NAGR, SGF, and CAN than in controls (P=0.003, P<0.001, P=0.002, P<0.0001, respectively) (Table 2, Figs. 1A and 2A and B). The highest proportions of freshly isolated CD40L+/CD4+ T cells among the patients were observed in recipients with CAN, but the difference was statistically significant only in comparison with recipients with SGF (P=0.022) (Fig. 2A and B). It is noteworthy that only in the group of graft recipients with CAN was the median percentage of freshly isolated CD40L+/CD4+ T cells similar to that found in C after ex vivo stimulation, while in the other groups of patients these values were significantly lower compared with C (AGR vs. C: P=0.015, NAGR vs. C: P=0.005 and SGF vs. C: P=0.001).

Table 2
Median percentage (and interquartile range) of peripheral blood CD3+/CD4+ cells expressing CD40L before and after 72 h of culture with anti-CD3+rIL-2 in all studied groups

Studied groups	Freshly isolated cells	Anti-CD3+rIL-2-stimulated cells
AGR (n=17)	2.15 (0.6 to 8.4) [a]	7.7 (2.5 to 9.4) [b]
NAGR (n=29)	3.2 (1.0 to 5.2) [a]	5.0 (2.5 to 11.0) [b, c]
CAN (n=10)	5.0 (3.4 to 12.3) [a, d]	3.4 (2.2 to 7.55) [b, e]
SGF (n=24)	2.3 (1.0 to 4.7) [a]	11.4 (5.4 to 22.8) [c]
C (n=27)	0.5 (0.3 to 1.3)	7.8 (5.2 to 10.6) [c]

Differences between groups were evaluated by the Kruskal–Wallis test. The dependent samples were compared by the Wilcoxon's test.
[a] $P\leq0.003$ vs. controls.
[b] $P<0.05$ vs. SGF.
[c] $P<0.05$ stimulated vs. freshly isolated cells.
[d] $P=0.022$ vs. SGF.
[e] $P=0.043$ vs. controls.

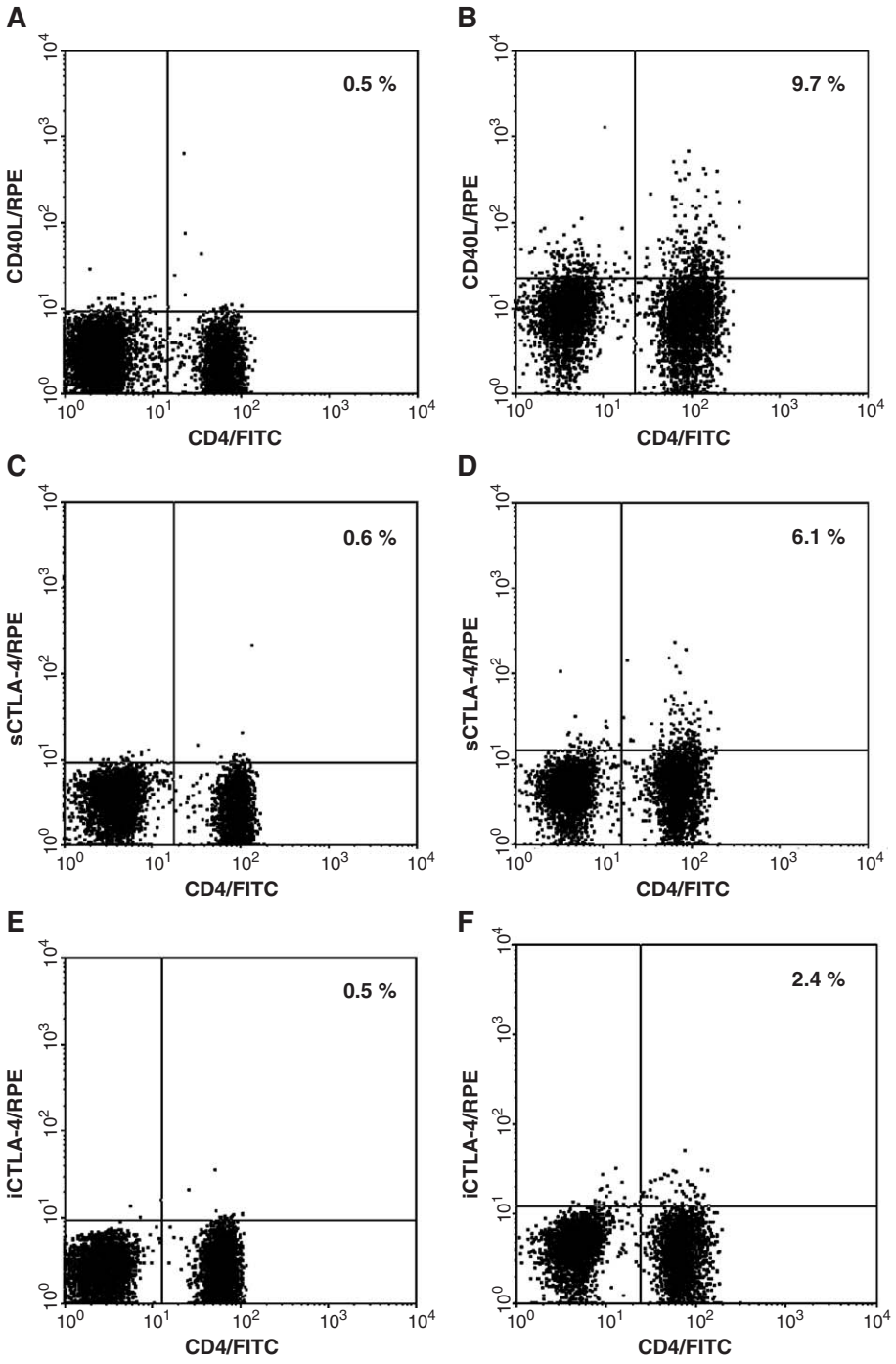

Fig. 1. The representative example of the CD40L, surface CTLA-4 (sCTLA-4) and intracellular CTLA-4 (iCTLA-4) expression on CD4+ T cells from healthy subjects before and after 72 h of culture with anti-CD3+rIL-2. The dot plots show the CD4/FITC vs. CD40L/RPE or CTLA-4/RPE distribution on CD3+ cells. (A, B) The CD40L expression on freshly isolated CD4+ T cells (A) and after 72 h of culture with anti-CD3+rIL-2 (B). (C, D) The sCTLA-4 expression on freshly isolated CD4+ T cells (C) and after 72 h of culture with anti-CD3+rIL-2 (D). (E, F) The iCTLA-4 expression on freshly isolated CD4+ T cells (E) and after 72 h of culture with anti-CD3 +rIL-2 (F).

In control cultures without stimulants, the proportions of CD40L+/CD4+ T cells in all the studied groups decreased to undetectable levels (not shown).

Ex vivo stimulation increased the median percentage of CD4+ T cells expressing CD40L in recipients with SGF ($P<0.001$) (Fig. 2B and D), with NAGR ($P=0.049$), and in C ($P<0.001$) (Fig. 1A and B). The highest median percentage of CD40L+/CD4+ T cells among the examined groups of graft recipients was observed in the group with SGF (vs. NAGR: $P=0.028$; vs. CAN: $P=0.019$; vs. AGR: $P=0.049$) (Table 2, Fig. 2C and D). In patients with NAGR, AGR, and SGF the median frequencies of CD40L+/CD4+ T cells were similar to that found in C, while in patients with CAN these values were significantly lower compared with C ($P=0.043$) (Figs. 1B and 2C).

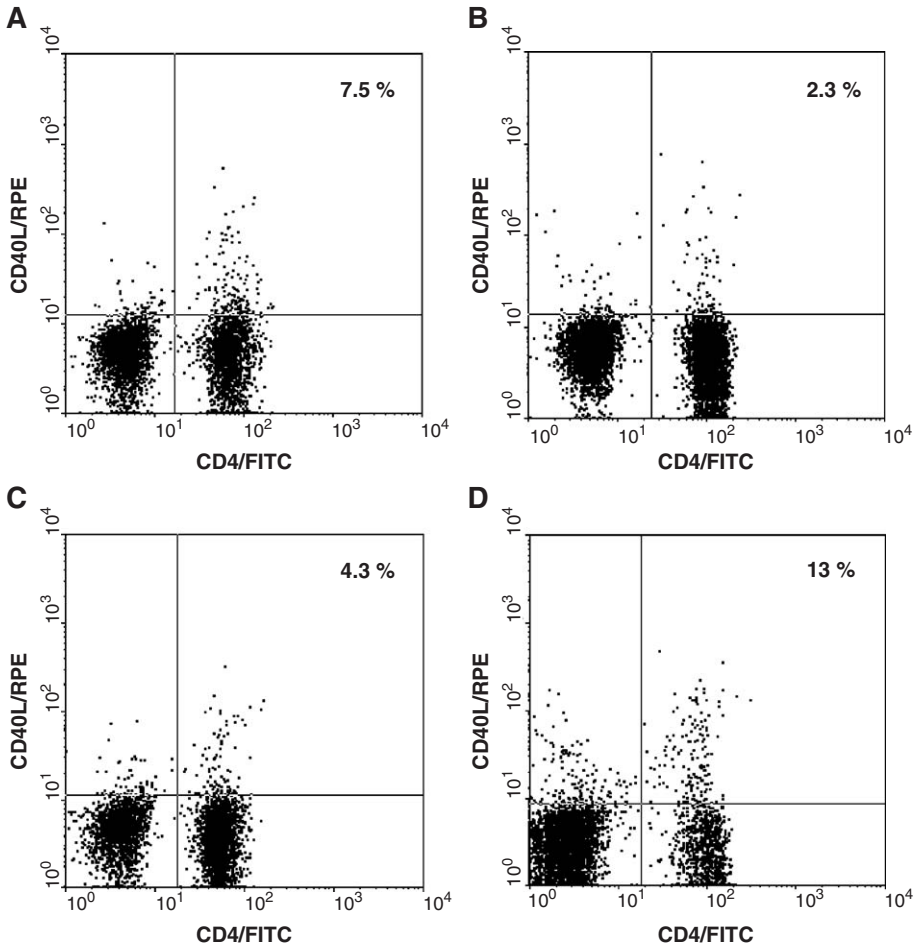

Fig. 2. The dot plots show representative data of the CD40L expression on peripheral blood CD4+ T cells from renal graft recipients with chronic allograft nephropathy (CAN) and with stable graft function (SGF) before and after 72 h of culture with anti-CD3+rIL-2. The dot plots show the CD4/FITC vs. CD40L/RPE distribution on CD3+ cells. (A, B) The CD40L expression on freshly isolated CD4+ T cells from patient with CAN (A) and with SGF (B). (C, D) The CD40L expression on CD4+ T cells from patient with CAN (C) and with SGF (D) after 72 h of culture with anti-CD3+rIL-2.

4.2. Expression of costimulatory CD28 molecule on freshly isolated and anti-CD3+rIL-2-stimulated CD4+ T cells in kidney graft recipients and controls

No significant differences in the frequencies of CD28+/CD4+ T cells of freshly drawn PB were found among the groups of kidney transplant recipients and the control group (Table 3).

Table 3
Median percentage (and interquartile range) of peripheral blood CD3+/CD4+ cells expressing CD28 before and after 72 h of culture with anti-CD3+rIL-2 in all studied groups

Studied groups	Freshly isolated cells	Anti-CD3+rIL-2-stimulated cells
AGR (n=17)	90.5 (38.0 to 98.0)	79.0 (24.0 to 97.0)
NAGR (n=29)	80.0 (41.0 to 97.0)	70.0 (34.0 to 95.0)
CAN (n=10)	95.5 (87.0 to 97.0)	88.0 (48.0 to 99.0)
SGF (n=24)	93.5 (86.5 to 98.0)	77.0 (33.5 to 96.0)[a]
C (n=27)	95.0 (89.0 to 97.0)	94.0 (71.0 to 98.0)

The dependent samples were compared by the Wilcoxon's test.
[a] P<0.005 stimulated vs. freshly isolated cells.

In control cultures without stimulants, the proportions of CD4+ T cells expressing CD28 did not change (not shown).

Anti-CD3+rIL-2 stimulation significantly decreased the median proportion of CD28+/CD4+ T cells in recipients with SGF (P=0.017) (Fig. 3), but did not change the median proportion of these cells in patients with AGR, NAGR, and CAN.

4.3. Expression of the surface CTLA-4 (sCTLA-4) molecule on freshly drawn and anti-CD3+rIL-2-stimulated CD4+ T lymphocytes from kidney graft recipients and controls

The proportions of sCTLA-4+/CD4+ T cells in PB among all graft recipients were comparable and statistically higher compared with C (AGR: P<0.001, NAGR: P<0.001, SGF: P<0.001, CAN: P<0.001) (Table 4, Figs. 1 C and 4A and B).

In the control cultures without stimulants, the proportions of sCTLA-4+/CD4+ T cells decreased to undetectable levels in all groups of graft recipients (not shown).

Anti-CD3+rIL-2 stimulation decreased the median proportion of these cells in patients with CAN (P=0.042) (Fig. 4A and C). In control subjects the median frequencies of sCTLA-4+/CD4+ T cells increased after ex vivo stimulation (P<0.001) (Fig. 1C and D). Comparison of the median percentages of ex vivo stimulated sCTLA-4+/CD4+ T cells

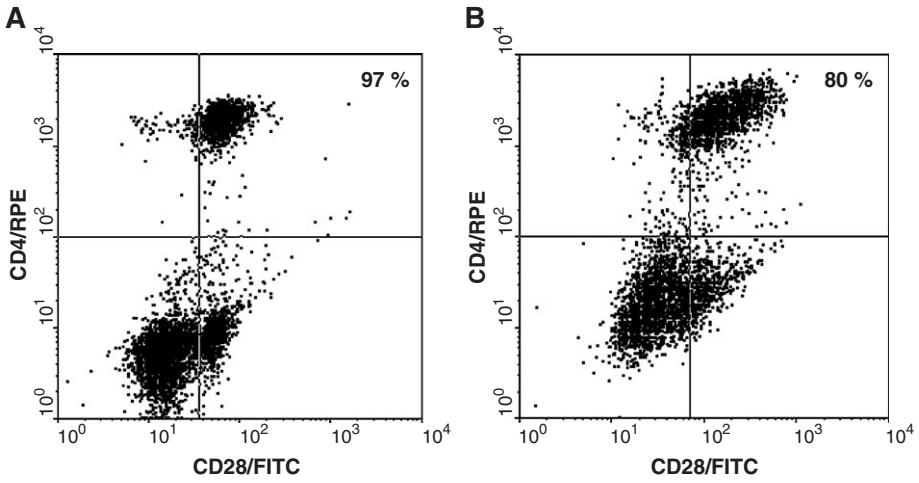

Fig. 3. The dot plots show representative data of the CD28 expression on peripheral blood CD4+ T cells from renal graft recipients with stable graft function (SGF) before and after 72 h of culture with anti-CD3+rIL-2. The dot plots show the CD28/FITC vs. CD4/RPE distribution on CD3+ cells. (A) The CD28 expression on freshly isolated CD4+ T cells. (B) The CD28 expression on CD4+ T after 72 h of culture with anti-CD3+rIL-2.

showed an sCTLA-4 expression in the group of kidney recipients with SGF similar to that found in C. In the groups of patients with AGR, NAGR, and CAN, the median proportions of these cells were significantly lower compared with C ($P=0.002$, $P=0.005$, $P=0.003$, respectively) and with patients with SGF ($P=0.008$, $P=0.049$, $P=0.009$, respectively) (Table 4, Fig. 4C and D).

4.4. Expression of the intracellular CTLA-4 (iCTLA-4) molecule in freshly isolated and anti-CD3+rIL-2-stimulated CD4+ T lymphocytes from kidney graft recipients and controls

The median percentages of freshly isolated iCTLA-4+/CD4+ T cells were significantly higher in all groups of graft recipients than in C (AGR: $P=0.02$; NAGR: $P<0.001$; SGF: $P<0.001$; CAN: $P<0.001$) (Table 5) (Figs. 1E and 5A and B). Although among the studied groups of patients the highest median proportion of iCTLA-4+/CD4+ T cells was found in the recipients with SGF, the differences were only statistically significant in comparison with AGR and CAN ($P<0.001$, $P=0.015$, respectively) (Fig. 5A and B). Moreover, iCTLA-4 expression in the group with NAGR was markedly higher than that observed in AGR ($P=0.02$).

Table 4
Median percentage (and interquartile range) of peripheral blood CD3+/CD4+cells expressing surface CTLA-4 before and after 72 h of culture with anti-CD3+rIL-2 in all studied groups

Studied groups	Freshly isolated cells	Anti-CD3+rIL-2-stimulated cells
AGR ($n=17$)	1.9 (1.4 to 9.85) [a]	1.5 (0.4 to 4.5) [b, c]
NAGR ($n=29$)	2.6 (1.75 to 10.6) [a]	1.8 (1.0 to 6.1) [b, c]
CAN ($n=10$)	3.75 (0.8 to 6.35) [a]	0.8 (0.4 to 1.8 [b, c, d]
SGF ($n=24$)	6.05 (2.4 to 12.45) [a]	4.0 (2.2 to 7.6)
C ($n=27$)	0.2 (0.1 to 0.3)	4.3 (3.7 to 5.6) [d]

Differences between groups were evaluated by Kruskal–Wallis test. The dependent samples were compared by the Wilcoxon's test.
[a] $P<0.001$ vs. controls.
[b] $P\leq0.005$ vs. controls.
[c] $P\leq0.05$ vs. SGF.
[d] $P\leq0.042$ stimulated vs. freshly isolated cells.

The percentages of CD4+ T cells expressing iCTLA-4 in control cultures in medium alone decreased to the levels obtained in control subjects before stimulation (not shown).

Ex vivo stimulation resulted in a significant increase in the frequency of iCTLA-4+/CD4+ T cells only in C ($P<0.001$) (Fig. 1E and F). Analysis of the proportions of ex vivo stimulated iCTLA-4+/CD4+ T cells revealed no significant differences among the four studied groups of graft recipients. In comparison with C, a significantly higher proportion of stimulated iCTLA-4+/CD4+ T cells was only found in kidney transplant patients with SGF ($P=0.002$) (Figs. 1F and 5D), while no differences were observed in the other groups of patients (Table 5).

5. Discussion

In this study we analyzed systematically for the first time the expressions of both costimulatory CD40L and CD28 and down-regulatory CTLA-4 molecules on PB CD4+ T cells before and after ex vivo stimulation in renal graft recipients in relation to the post-transplantation clinical course. We found that the median frequency of freshly drawn PB CD4+ T cells expressing CD28 molecule, which is constitutively expressed on CD4+ T cells, was not different in the recipients and control subjects. In contrast, elevated median percentages of CD4+ T cells expressing CD40L and both sCTLA-4 and iCTLA-4 in all kidney graft recipients compared with controls were shown. As CD40L and CTLA-4 are expressed on T cells after activation, their increased spontaneous expression probably reflects the extent of in vivo CD4+ T-cell activation. This hypothesis can be supported by the fact that a 72-h culture of these cells in culture medium alone resulted in a decrease in the expressions of these molecules to very low levels (not shown).

Several differences in the expressions of these molecules on PB CD4+ T cells before and after ex vivo stimulation were found among the studied groups of graft recipients. The most apparent differences were observed between patients with SGF and CAN. In recipients with uneventful post-transplant course, lasting at least 18 months, the median percentage of freshly

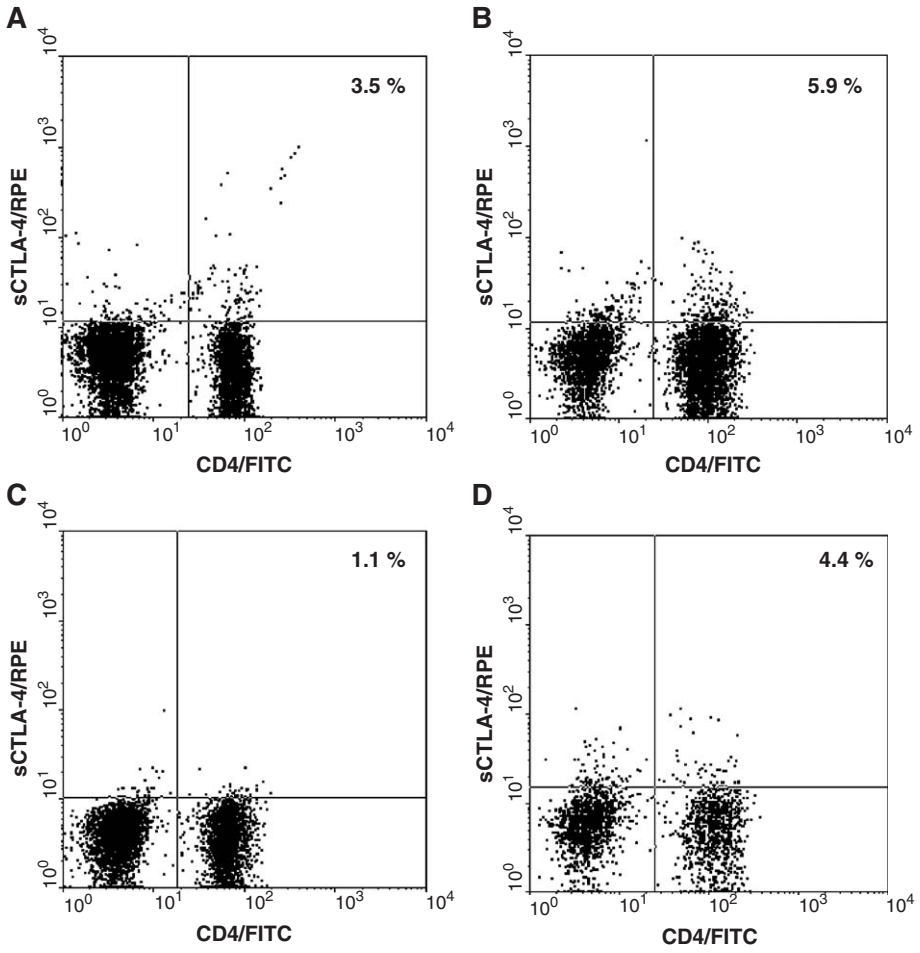

Fig. 4. The dot plots show representative data of the surface CTLA-4 (sCTLA-4) expression on peripheral blood CD4+ T cells from renal graft recipients with chronic allograft nephropathy (CAN) and with stable graft function (SGF) before and after 72 h of culture with anti-CD3+rIL-2. The dot plots show the CD4/FITC vs. CTLA-4/RPE distribution on CD3+ cells. (A, B) The sCTLA-4 expression on freshly isolated CD4+ T cells from patient with CAN (A) and with SGF (B). (C, D) The sCTLA-4 expression on CD4+ T cells from patient with CAN (C) and with SGF (D) after 72 h of culture with anti-CD3+rIL-2.

drawn CD4+ T cells expressing CD40L was significantly lower than in patients with CAN. In contrast, the median proportion of CD4+ T cells expressing iCTLA4 molecule was significantly higher and those expressing sCTLA-4 was also higher, although not significantly so when compared with patients with CAN. Ex vivo stimulation up-regulated the median percentage of CD40L +/CD4+ T cells in patients with SGF to levels similar to that observed in controls and higher than those found in CAN and other groups of graft recipients. Interestingly, only in the recipients with SGF did ex vivo re-stimulation result in a significant decrease in the frequency of CD28+/CD4+ T cells. It was previously established that CD28 expression is physiologically down-regulated after 24 h at both the mRNA and protein levels and returns to prestimulation levels thereafter. It has been also shown that transient down-regulation of CD28 expression is a strong inducer of CTLA-4 gene expression [8,20]. Previously we found that, in fact, patients with down-regulated levels of CD28 due to stimulation exhibited elevated CTLA-4 expression [17]. The decreased levels of CD28 after 72 h of stimulation in SGF patients we observed in this study may be responsible for the maintenance of stable, high levels of either sCTLA-4 or iCTLA-4 expression on CD4+ T cells after ex vivo re-stimulation in these patients. In fact, the median percentage of sCTLA-4+/CD4+ T cells after ex vivo stimulation was significantly higher in SGF compared with other groups of graft recipients, which may lead to the strongest down-regulation of

Table 5
Median percentage (and interquartile range) of peripheral blood CD3+/CD4 +cells expressing intracellular CTLA-4 before and after 72 h of culture with anti-CD3+rIL-2 in all studied groups

Studied groups	Freshly isolated cells	Anti-CD3+rIL-2-stimulated cells
AGR (n=17)	0.8 (0.4 to 1.8)[a, b]	3.5 (2.55 to 6.2)
NAGR (n=29)	1.95 (1.05 to 8.05)[a, c]	4.0 (1.3 to 10.2)
CAN (n=10)	1.3 (0.9 to 2.0)[a, b]	4.85 (2.45 to 7.3)
SGF (n=24)	4.5 (1.6 to 7.9)[a, c]	6.7 (3.15 to 14.65)[d]
C (n=27)	0.3 (0.2 to 0.5)	2.1 (1.2 to 3.8)[e]

Differences between groups were evaluated by Kruskal–Wallis test. The dependent samples were compared by the Wilcoxon's test.
[a] $P \leq 0.02$ vs. controls.
[b] P=0.015 vs. SGF.
[c] P=0.02 vs. AGR.
[d] P=0.002 vs. controls.
[e] $P \leq 0.008$ stimulated vs. freshly isolated cells.

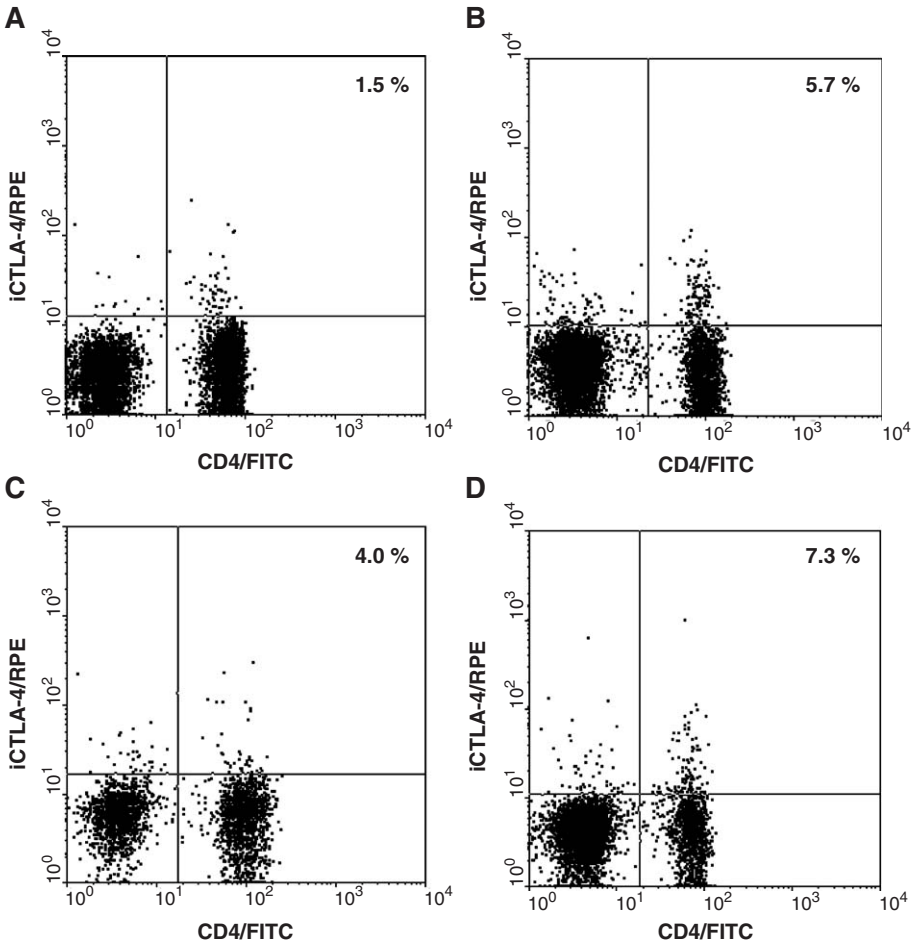

Fig. 5. The dot plots show representative data of the intracellular CTLA-4 (iCTLA-4) expression on peripheral blood CD4+ T cells from renal graft recipients with chronic allograft nephropathy (CAN) and with stable graft function (SGF) before and after 72 h of culture with anti-CD3+rIL-2. The dot plots show the CD4/FITC vs. CTLA-4/RPE distribution on CD3+ cells. (A, B) The iCTLA-4 expression on freshly isolated CD4+ T cells from patient with CAN (A) and with SGF (B). (C, D) The iCTLA-4 expression on CD4+ T cells from patient with CAN (C) and with SGF (D) after 72 h of culture with anti-CD3+rIL-2.

the alloimmune responses and long-term allograft acceptance in this patients.

The highest median proportion of freshly drawn CD4+ T cells expressing CD40L were observed in patients with CAN, although the difference was statistically significant only in comparison with SGF. Since ex vivo stimulation did not change the median level of CD40L expression, we assume that these cells are maximally stimulated in vivo and do not respond to re-stimulation.

In addition, in patients with CAN, in contrast to the other groups of graft recipients, a marked decrease in the down-regulatory sCTLA-4 molecule on CD4+ T cells after ex vivo stimulation was noted. The mechanism leading to this down-regulation of sCTLA-4 expression is not clear. It was previously shown that CTLA-4 protein is located primarily in intracellular vesicles. During T-cell activation, CTLA-4 molecules are transported to the cell surface and subsequent rapid internalization of this receptor is observed [21]. Since the level of iCTLA-4 expression in CAN did not significantly change after ex vivo stimulation, we suggest that the down-regulation of sCTLA-4 expression in these patients may result from disturbed recycling of iCTLA-4 to the cell surface. Consistent with observations that only sCTLA-4 signals, but not iCTLA-4 ones, negatively regulate activated T cells, our novel findings point to the possibility that the marked decrease in the proportion of sCTLA-4+/CD4+ PB T cells after re-stimulation may lead to diminished sCTLA-4-mediated inhibition of T-cell alloresponses and thereby promote graft rejection in CAN. In fact, studies with blocking antibodies to CTLA-4 have shown that CTLA-4 blockade enhances acute allograft rejection even in CD28-deficient mice [22].

The differences in the studied molecule expressions in patients tested in the early post-renal transplantation period, i.e. AGR and NAGR, were less prominent than those found between CAN and SGF, probably because of the modifying effect of high-dose immunosuppressive treatment in these patients. Non-significantly higher sCTLA-4 and significantly higher iCTLA-4 median proportions in freshly drawn CD4+ T cells were observed in patients with NAGR compared with AGR. In addition, ex vivo stimulation significantly up-regulated CD40L expression in patients with NAGR, again indicating the possible involvement of these molecules in the post-transplantation clinical course.

In summary, our results clearly show a relationship between the pattern of co-stimulatory and down-regulatory molecule expression and the clinical course after renal transplantation, most strongly pronounced between SGF and CAN patients. This study demonstrates for the first time non-significantly higher sCTLA-4, significantly higher iCTLA-4, and markedly lower CD40L expressions on freshly drawn PB CD4+ T cells in patients with SGF compared with CAN, reflecting different types of immune response to allograft in these patients. In addition, PB CD4+ T cells from patients with SGF showed a higher potential to express sCTLA-4 and CD40L molecule and to down-regulate CD28 molecule in response to ex vivo stimulation than those from patients with CAN. Similar but less prominent differences in the expressions of these molecules in PB CD4+ T cells before and after ex vivo stimulation were observed between patients in the early post-transplant course (AGR and NAGR). The differences between the SGF and CAN groups shown in our study are of a potential clinical relevance. CAN is, besides the death of the recipient with a functioning graft, the most common cause of renal transplant loss. The key issue in the approach to these patients with slowly but continuously deteriorating allograft function is the proper distinction between the immunologic chronic rejection process and long-term calcineurin inhibitor nephrotoxicity. Recipients showing a stepwise decline in allograft function but simultaneously exhibiting the pattern of costimulatory (CD40L) and inhibitory (CTLA-4) expression associated in our study with long-standing normal kidney allograft function (i.e. as in the SGF group) could be appropriate candidates for calcineurin minimization or withdrawal protocols.

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