RESEARCH ARTICLE

Enzyme-Linked Immunosorbent Spot (ELISpot) monitoring of cytokine-producing cells for the prediction of acute rejection in renal transplant patients

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ABSTRACT. The purpose of this study was to evaluate T-cell immunity markers using serial post-transplantation monitoring of cytokine-producing cells during the first post-transplant months for the prediction of acute rejection and potentially chronic rejection of kidney allograft. We followed 57 kidney allograft recipients for meanly 3 years post-transplantation. Blood samples were collected pre-transplant, 2, 4 and 12 weeks post-transplant. The frequencies of IL-10-, IL-17- and IFN-γ-producing cells were determined in all time-points using ELISPOT assay. The results of ELISpot monitoring and levels of IL-23 and TGF-β were compared between recipients with acute (n = 12) or chronic rejection episodes and patients with stable graft function (n = 45). In all post-transplant time-points, significantly high frequencies of IFN-γ- and IL-17-producing cells and low frequency of IL-10-producing cells were observed in rejection group *versus* patients with stable graft function ($P < 0.0001$). The ROC curve analysis for determining the reliability of cytokine-producing cells for the prediction of acute rejection revealed that AUC was 0.046 for IL-10 ($P < 0.001$), 0.927 for IL-17 ($P < 0.001$) and 0.929 for INF-γ-producing cells ($P < 0.001$). Our results indicate that analyzing the frequencies of INF-γ/IL-10/IL-17-producing cells may define a reliable panel for the prediction of acute rejection within the first post-transplant year which could also be applicable for the prediction of chronic rejection episodes.

Key words: kidney transplantation, rejection, ELISpot, cytokines, prognosis

Acute rejection (AR) as the main predictor of chronic rejection is an important cause of graft loss after renal transplantation even in the era of modern immunosuppression [1, 2]. The current gold standard method for the diagnosis of rejection is renal biopsy which is invasive and risky for the patients firstly and secondly can be inaccurate due to the targeting of non-representative area for rejection [3]. Therefore, improving the diagnosis of rejection using non-invasive methods and more importantly the prediction of rejection before overt graft damages have been extensively considered in recent years [3, 4].

Despite outstanding progresses in assessing the humoral alloimmune responses against alloantigens of renal graft and accordingly reduced rate of antibody-mediated rejection, monitoring of T-cell alloimmune responses for the prediction of T cell-mediated rejection is still under debate [5]. It is well known that T cells have central role in both acute and chronic graft rejection [6] and contribution of the proinflammatory cytokines produced by T cells in rejection of allograft has been well documented [1, 7, 8]. Nowadays, interferon-gamma (IFN-γ) Enzyme-Linked Immunosorbent Spot (ELISPOT) testing before and after transplantation gives us valuable information about immune status in the allograft recipients [9]. Interleukin-2 (IL-2) and IFN-γ as prototypes for Th1 cytokines have been shown to increase during rejection process and both cytokines can provide precious information regarding kidney allograft prognosis [10, 11]. Also, investigations on Th17-Tregs axis in preclinical and clinical studies have implicated that regulatory T cells can be converted into Th17 cells during inflammatory conditions (*e.g.* allograft rejection) and this plasticity feature of Tregs is estabilished in the presence of transforming grows factor-beta (TGF-β) and IL-23/IL-21 that could be

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induced by IL-1 and/or IL-6 cytokines [12]. On the other hand, increasing levels of IL-10 may have a protective role toward maintaining hyporesponsiveness and cause good graft function [13]. Hence, evaluation of cytokine profiles representative for regulatory T cells (*e.g.* TGF-β and IL-10) and Th17 (*e.g.* IL-17 and IL-23) could be worthwhile along with Th1 and humoral immunity monitoring in transplant patients [23]. It has been also demonstrated that urinary-cell mRNA levels could be diagnostic and prognostic for acute rejection of kidney allografts but this test is $\sim$ 80% specific and sensitive [3, 4]. Nevertheless, there is not yet any specific early post-transplant immunological assay to predict kidney allograft outcome and hence, further investigations are required to define an accurate and more clinically relevant immune monitoring assay for management of kidney transplant patients [9]. Despite growing body of evidences about central role of Th cells and their cytokines in alloimmune responses against allograft, the exact pathogenic effects of these mediators of cellular immunity in AR are arguable [14]. In this milieu, evaluation of cytokine profiles as prognostic measures might be useful because they are noninvasive and more importantly could help us to improve the care of allograft recipients by selection and adjustment of immunosuppressive drugs based on more fine-tune immune monitoring approaches.

We previously demonstrated that the serial monitoring of IFN-γ-producing donor reactive cells within the first months post-transplant is a clinically feasible approach for discriminating recipients at risk for developing AR of kidney allograft from those with stable graft function [15]. In the present study, we have completed our previous data by analyzing more T-cell immune markers pre and post-transplantation by using the same protocol (ELISPOT) and considering longer follow-up data (meanly 3 years) to find out a more accurate and potentially combinational prognostic signature for acute rejection and possibly for chronic rejection episodes in renal transplant patients.

PATIENTS AND METHODS

Fifty-seven primary kidney allograft recipients with background diseases including hypertension, glomerulonephritis, diabetes, nephrotoxicity and kidney stone were enrolled in this multi-center prospective study between September 2011 and October 2015. Enrolled patients received living donor renal allografts either from related (n = 20) or unrelated donors (n = 37). All patients provided a written informed consent and the study was specifically approved by the institutional ethics committee. Maintenance dose of immunosuppression consisted of cyclosporine A, mycophenolate mofetil (MMF) and methyl prednisolone without the implementation of any antibody induction therapy. Renal biopsies were performed in those patients with suspected rejection episodes, rise in serum creatinine by $\geq$ 0.3 mg/dl from the baseline in two consecutive days along with clinical symptoms and biopsy results were graded using Banff criteria scoring [16]. Delayed graft function was defined as a requirement for dialysis within the first week post-transplant due to increased serum creatinine levels after ruling out of other causes of graft dysfunction. Patients with no history of clinical and/or biopsy-proven rejection and with good graft functioning based on serum creatinine level ($<$1.5 mg/dL) were considered as stable graft function. During the first year follow-up, patients were subdivided into two groups including patients who experienced acute rejection (n = 12) and those with stable functioning of the graft (n = 45). Both groups of the patients were followed prospectively for meanly 3 years to determine the prognostic value of early sequential monitoring of different cytokine-producing cells for acute rejection and even chronic rejection episodes.

HLA typing and antibody testing

By using conventional complement dependent cytotoxicity (CDC) method, not only percentage of pre-transplant panel-reactive antibody (PRA) was calculated and but also WBC cross-match was performed. HLA-DNA typing was done by using standard PCR-SSP technique for all donors and recipients to determine the HLA-A, B and DRB1 alleles (HLA-ABDR Low Resolution kit, Biotest, Germany).

Enzyme-linked immunosorbent spot (ELISPOT) assay

Peripheral blood samples were collected in EDTA containing tubes before transplantation and 2, 4 and 12 weeks after transplantation in three University hospitals (Labbafi Nejad, Imam Khomeini and Baghiatallah). Peripheral blood mononuclear cells (PBMCs) were isolated on standard ficoll (Amersham, Germany) density gradient preparation and then frozen in media containing FBS (Gibco) and DMSO (Sigma-Aldrich-USA) in 9:1 ratio till ELISPOT assay. The ELISPOT method was performed according to the manufacturer's instructions (Human IFN-γ/IL-10/IL-17 Elispot Ready-Set-Go eBioscience, Vienna, Austria). Briefly, 96-well ELISPOT plates were coated with 100μL/well of diluted capture antibodies for IFN-γ, IL-10 and IL-17 separately and incubated overnight at 4°C. After 2 times washing with 200μL/well coating buffer, plates were blocked with RPMI 1640 containing L-glutamine plus penicillin/streptomycin for 1 hour at room temperature. Afterwards, the medium was aspirated and a total of 1×10^5 responder PBMCs in 100 μL of complete RPMI-1640 containing 10% human serum (Gibco) and L-glutamine plus penicilin/streptomycin were placed to each well in a duplicate manner. PBMCs were stimulated with phytohemaglutinin (PHA, Sigma, Munich, Germany) at final concentration of 1 μg/mL of RPMI medium as positive controls and donor inactivated lymphocytes by mitomycin (Sigma, M0503, Germany) 2×10^4 cells/well, to serve as test. Negative controls were also run in duplicates using responder cells alone in complete medium without stimulation. Plates were incubated at 37°C for 48 hours and then, supernatants were collected for measurement of TGF-β and IL-23 by ELISA method. Next, plates were washed and biotinylated detection antibodies for IFN-γ, IL-10 and IL-17 were added to each plate separately. After 2 hours incubation at room temperature and following wash steps, avidin-horse radish peroxidase was added for 45 minutes at room temperature and then, the spots were developed using freshly prepared ACE (3-amino-9-ethylcarbazole) reagent. Finally, by using a dissecting microscope, resulting spots were

Table 1
Demographic data and clinical characteristics for all patients.

Parameters	**Acute rejection group (*n* = 12)**	**Stable graft group (*n* = 45)**
Age in years (mean ± SD)	41.33 ± 7.49	45.24 ± 13.38
Female/male	6 (50.0%) /6 (50.0%)	18 (40.0%) /27(60.0%)
Number of HLA mismatches (HLA-A, B, DR)		
0 - 2 mM	2 (16.7%)	4 (8.9%)
3 - 5 mM	6 (50.0%)	28 (62.2%)
6 mM	4 (33.3%)	13 (28.9%)
CMV status		
Neg	9(75%)	32(71.11%)
Pos	3(25%)	13(28.89)
Immunosuppressive regimen		
Cyclosporine A	Adjust dose	Adjust dose
Mycophenolate Mofetil	Adjust dose	Adjust dose
Methyl prednisolone	Adjust dose	Adjust dose
Serum Cr levels (mg/dl) (Mean ± SD)		
Day 0	7.90 ± 2.26	6.52 ± 2.63
Day14	1.59 ± 0.76	1.57 ± 0.85
Day 28	1.79 ± 1.20	1.59 ± 0.46
Week 12	1.72 ± 1.01	1.59 ± 0.54
Week 24	1.63 ± 0.82	1.43 ± 0.55
Week 36	1.65 ± 0.93	1.60 ± 0.99
Week 48	1.86 ± 0.95	1.79 ± 1.00
Month 36*	1.72 ± 0.53	1.24 ± 0.47

HLA: human leukocyte antigens, CMV: Cytomegalovirus, Cr: creatinine. *: Comparison of serum creatinine levels after 3 years follow-up, SGF (*n* = 38) *vs.* ARE (*n* = 11), *P* = 0.002.

counted and the frequencies of cytokine-producing cells were calculated by subtracting the number of spots in negative control wells from the values counted in the test wells.

TGF-β and IL-23 measurement by enzyme-linked immunosorbent assay (ELISA)

These assays were carried out on supernatants collected from the ELISPOT plates (above described). The assay procedures were performed as per manufacturer's instructions (Human IL-23 and Human/Mouse TGF-β ELISA Ready-Set-Go, eBioscience, Vienna, Austria).

Statistical analysis

Descriptive statistics are presented as means and standard deviations. We tested all quantitative variables for normality with Shapiro-Wilk test. Friedman test was used to compare the changing in cytokines levels in two groups. Repeated measures ANOVA were conducted to compare the levels of cytokines. Logistic regression was performed to find out the significant determinants of AR. Factor analysis was used to build a set of more manageable variables and to decide which criteria can detect acute rejection. Receiver Operating Characteristic (ROC) curve was used to figure out the ability of factors to prognose acute rejection. Area under the curve (AUC) shows the prognostic value of factors. We considered P value < 0.05 as statistically significant. Data were analyzed using SPSS Version.16.0 for Windows.

RESULTS

A total of 57 renal transplant patients, 33 men and 24 women, with mean age of 44.4 ± 12.4 years old were enrolled in this prospective study. Patients' demographics are shown in *table 1*. Twenty of the 57 patients received allografts from living related donors and 37 were transplanted from living unrelated donors. All patients except four cases received HLA-mismatched renal transplants. During one-year follow-up, 45 patients showed stable graft functions 12 patients were diagnosed with acute rejection episodes (ARE). ARE was diagnosed based on allograft dysfunction as judged by rise in the serum creatinine levels along with defined clinical symptoms. Among 12 cases with ARE, cause biopsy was performed for 3 cases during the first 6- month post-transplant that revealed 1 with acute cellular rejection (Grade IB) and 2 with antibody-mediated rejection (C4d+). ARE was reversible in these patients by using steroid pulse therapy (500-1000 mg methyl prednisolone daily for 3 days). The remaining 9 patients showed rejection episodes without either protocol or cause biopsies and they were treated with increased baseline immunosuppression. However, the rejection episodes were diagnosed primarily after blood sample collection for immune monitoring except for 2 cases that experienced rejection episodes before month 2 post-transplantation. We observed lower but insignificant levels of serum creatinine were observed in patients with stable graft function compared to those with acute rejection. The only significant difference for serum creatinine levels between both groups was observed at month 36-post-transplantation ($P = 0.002$, *table 1*).

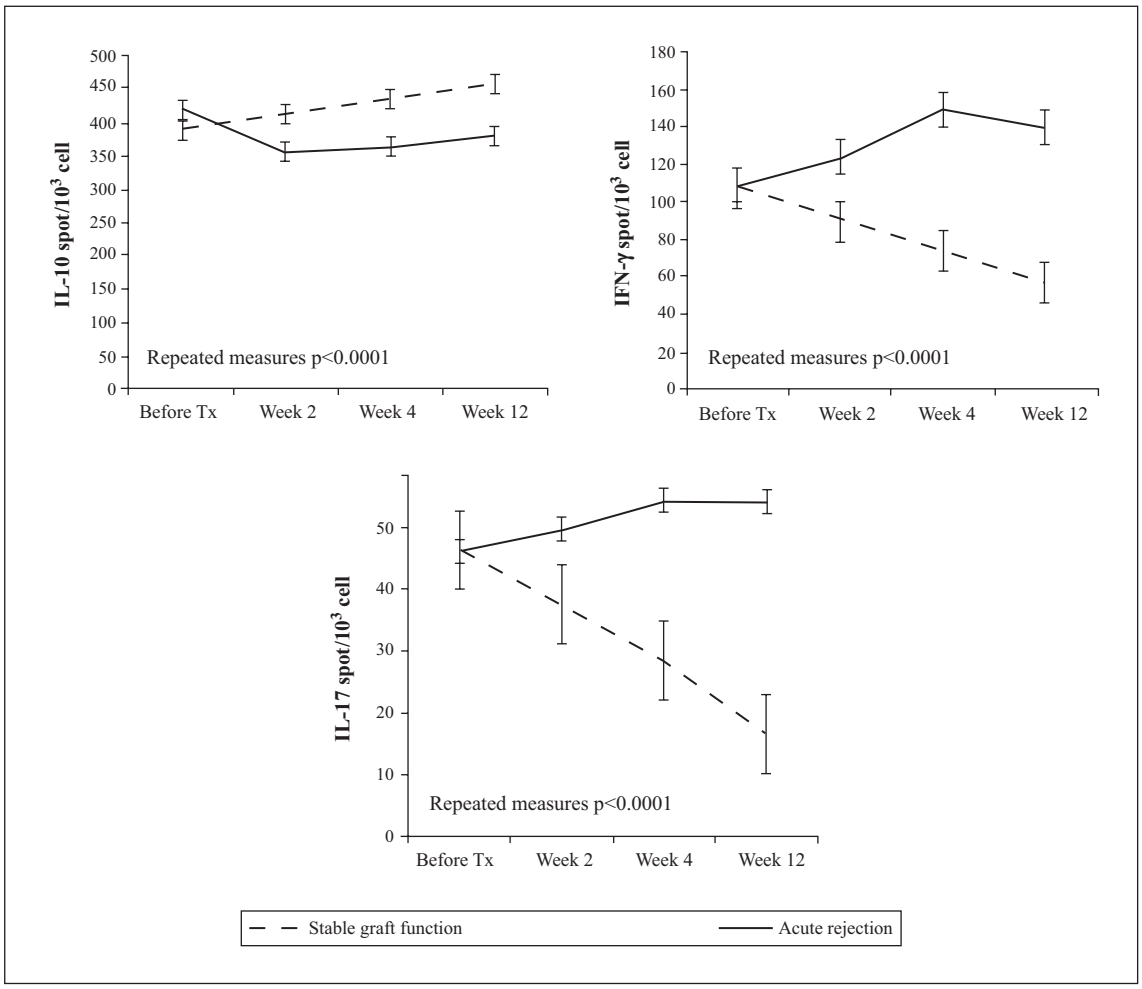


Figure 1
Comparison of the mean ELISPOT frequencies of donor-reactive IFN-γ, IL-10 and IL-17 producing cells at different time frames before and after transplantation (weeks 2, 4 and 12) in both groups of the patients.

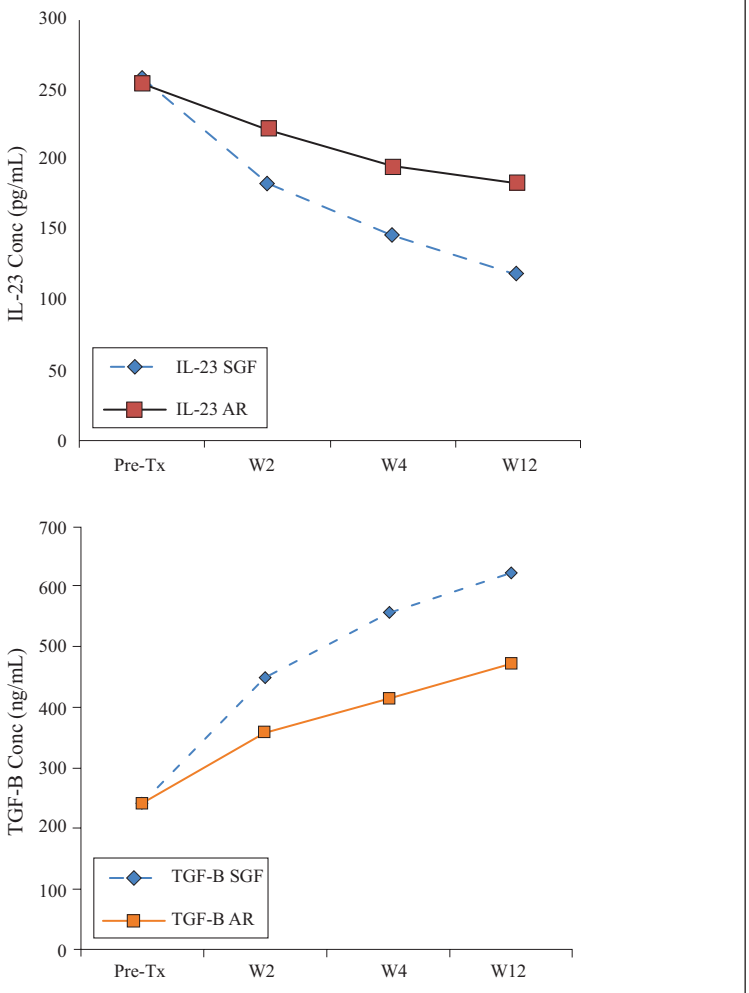
Figure 1 shows the mean ELISPOT frequencies of donor-reactive IFN-γ-, IL-10- and IL-17-producing cells at different time-frame pre and post-transplantation (weeks 2, 4 and 12) in both groups of the patients. We found significantly higher frequencies of IFN-γ- and IL-17-producing cells as well as low frequency of IL-10-producing cells in rejection group of patients compared to those with stable graft function in all post-transplant time-points ($P < 0.0001$, *figure 1*). Additionally, IL-23 and TGF-β levels in supernatants of cell culture medium of ELISPOT assays were significantly higher and lower respectively in rejection group than SGF group of the patients in 3 time points after transplantation ($P < 0.01$, *figure 2*).

Prospectively, three years follow-up of these patients revealed that 8 patients had reversible chronic rejection episodes, 6 biopsy-proved (4 humoral and 2 cellular rejections) and with history of ARE and 2 from SGF group. Also, 1 death with normal graft functioning was observed at month 28 post-transplantation. However, 5 patients were excluded from the analyses due to unavailable follow up data. We observed a significant increase in frequencies of IFN-γ- and IL-17-producing cells (*figure 3*), elevated IL-23 and decreased TGF-β levels in supernatants of cell culture medium of ELISPOT assays (*figure 4*) in patients who experienced chronic rejection episodes (CRE) compared to those with SGF. No significant differences were observed for the frequencies of IL-10-producing cells between both groups of the patients based on the occurrence of chronic rejection episodes (*figure 3*).

However, alterations in IL-10 levels were significantly correlated with recipients ages ($P = 0.041$). There was also a significant relation between changes in IL-23 levels and CMV infection ($P = 0.044$). No significant correlation was observed between progressive changes in the studied markers, gender, number of HLA mismatches and donor sources.

Univariate logistic regression analysis was conducted to determine the association between frequencies of cytokine-producing cells and occurrence of acute rejection. This analysis revealed that one unit of increase in IL-10 could cause 8.7% decrease in the risk of AR ($P = 0.002$), while by increasing of IL-17 and IFN-γ the risk becomes 19.9% ($P < 0.001$) and 8.3% ($P = 0.002$) higher for AR respectively. Also, IL-23 showed marginally significant changes for AR risk estimation ($P = 0.056$, *table 2*).

Receiver Operating Characteristic (ROC) analysis was implemented to determine the sensitivity and specificity of the frequencies of cytokine-producing cells for the detection of acute rejection (*figure 5*). The calculated area under curve (AUC) was 0.046 for IL-10-producing cells ($P < 0.001$), 0.927 for IL-17-producing cells ($P < 0.001$), 0.896 for IL-23 levels ($P < 0.001$) and 0.929 for INF-γ-producing cells ($P < 0.001$). Although IL-10 did not have suitable sensitivity and specificity for the



Time-points	IL-23 (pg/mL)			TGF-β (ng/mL)		
	SGF	ARE	*P value*	SGF	ARE	*P value*
Pre-Tx	257.9 ± 16.2	253.3 ± 17.6	*0.42*	243.6 ± 16.1	241.1 ± 21.5	*0.70*
Week 2	182.9 ± 8.5	212.3 ± 44.9	*0.01*	451.5 ± 25.6	358.5 ± 116.5	*0.01*
Week 4	146.3 ± 8.3	195.1 ± 58.2	*0.01*	556.6 ± 30.3	416.2 ± 132.6	*0.003*
Week 12	118.2 ± 7.9	183.5 ± 81.3	*0.01*	623.7 ± 20.2	473.6 ± 179.4	*0.01*

Figure 2

IL-23 and TGF-β levels in the supernatants of cell culture medium of ELISPOT assays in recipients with acute rejection (ARE, $n = 12$) and recipients with stable graft function (SGF, $n = 45$). Significant differences were observed in all 3 time points post-transplant for both cytokines ($P < 0.01$).

diagnosis of AR, a reliable prognostic power for differentiating AR can be provided by IL-10<400 Spots/10^5 Cells with IL-17>40 Spots/10^5 Cells, IFN-γ >100 Spots/10^5 Cells (ELISPOT) and IL-23>200 pg/mL (ELISA) on week 4 post-transplantation (Sensitivity = 83.33%, Specificity = 100%, Positive Predictive Value (PPV) = 100% and Negative Predictive Value (NPV) = 95.74%, *table 3*).

DISCUSSION

Early post-transplant immune monitoring may not only be predictive for long-term outcomes of kidney allograft, but also could be useful for identification of patients at high risk of acute rejection [17, 18]. This, in turn, will lead to individualized immunosuppressive therapy and improved long-term graft function as well as graft survival [19]. To achieve this major goal in clinical transplantation, defining reliable predictive biomarkers, particularly immune markers for outcomes of kidney allograft, has become a priority for the transplant community [20]. Several studies have shown that the frequencies of pre- and post-transplant IFN-γ-producing donor-reactive cells as determined by ELISpot assay correlate with kidney allograft outcome especially within the first year post-transplantation [6, 20-22]. In this study, we have updated the results from our previous study (14) by conducting the study on the same set of patients as the previous study and by collecting longer follow-up data and assessing more immune markers including IL-17, IL-23 and IL-10 as representative cytokines for T-cell immunity against alloantigens in kidney allograft. We observed significantly high frequency of IL-17-producing cells along with low frequency of IL-10-producing cells at weeks 2, 4 and 12

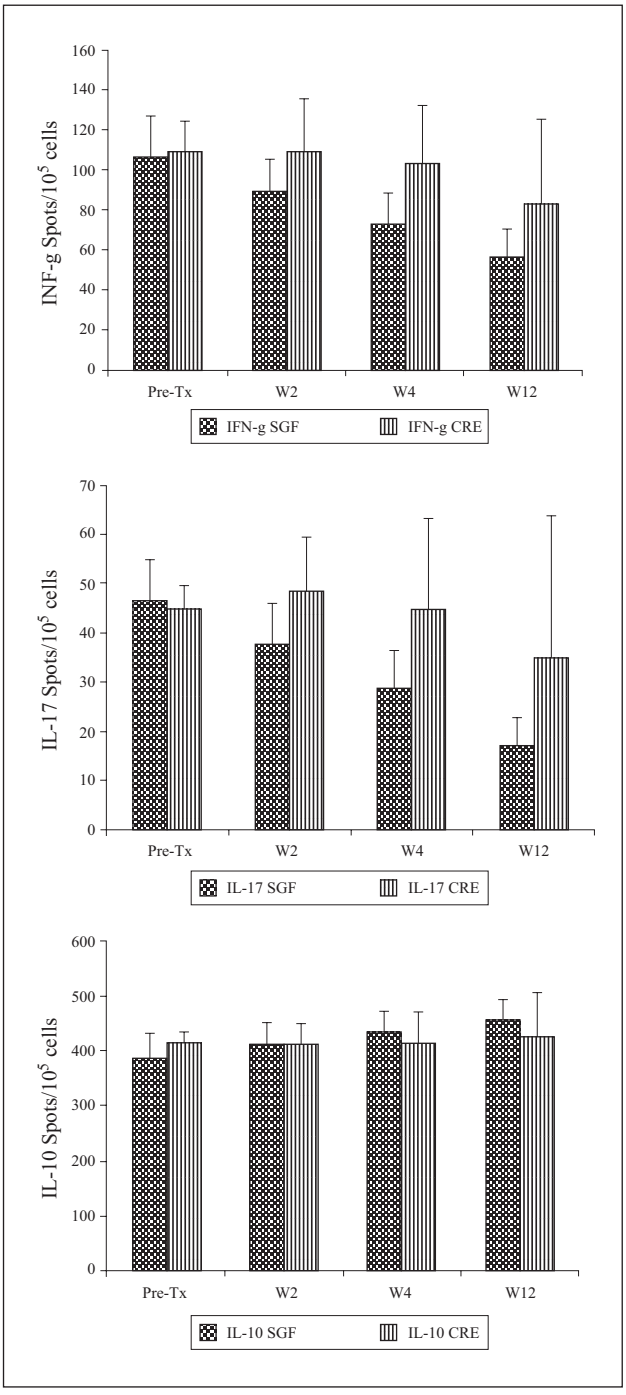


Figure 3
Comparison of the mean ELISPOT frequencies of donor-reactive IFN-γ, IL-10 and IL-17 producing cells at different time frames pre- and post-transplantation (weeks 2, 4 and 12) in patients with chronic rejection episodes (CRE, n = 8) and stable graft function (SGF, n = 43). Significant differences were observed in all 3 time points post-transplant between CRE and SGF groups of the patients for frequencies of IFN-γ and IL-17 producing cells but not for IL-10-producing cells.

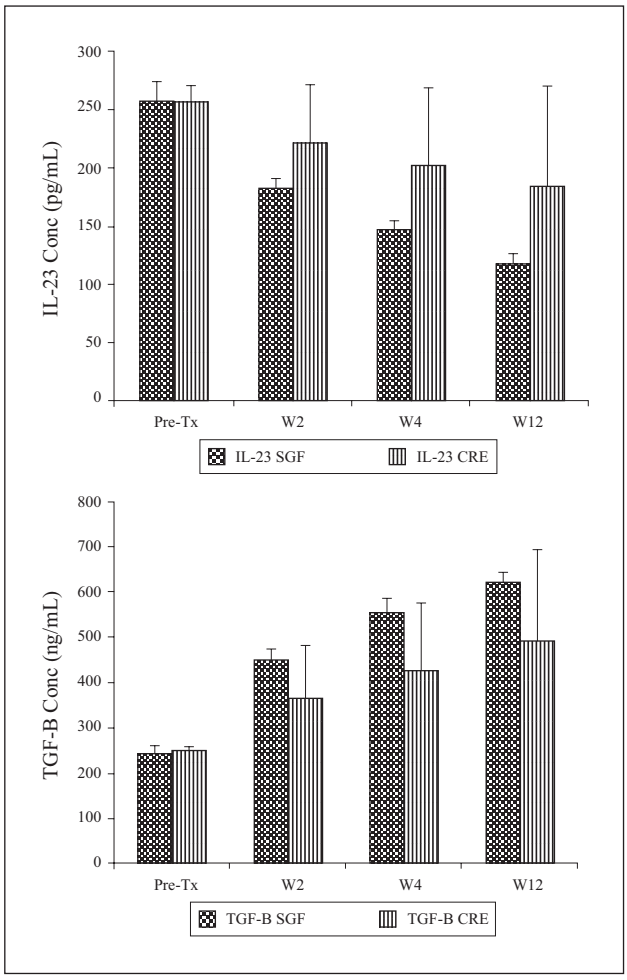


Figure 4
IL-23 and TGF-β levels in the supernatants of cell culture medium of ELISPOT assays in recipients with chronic rejection episodes (CRE, n = 8) and those with stable graft function (SGF, n = 43). Significant differences were observed in all 3 time points post-transplant for both cytokines ($P < 0.0001$).

post-transplant in patients with acute rejection episodes compared to those with stable graft function. Additionally, measurement of IL-23 and TGF-β levels in the supernatants obtained during ELISpot assay clearly showed increased IL-23 and decreased TGF-β levels in the rejection group of the patients *versus* the SGF group in all 3 time points post-transplantation. Remarkably, we found significant correlations between the frequencies of IFN-γ and IL-17-producing cells during first post-transplant month and occurrence of chronic rejection episodes in the second and third years of post-transplantation (*figures 3 and 4*). In addition, a similar correlation was observed between the alterations in IL-23 and TGF-β levels and CRE during 3 years of follow-up.

We observed that using cut-off levels based on AUC and ROC curve analysis, frequencies of IFN-γ, IL-17 and IL-10-producing cells and concentration of IL-23 in the culture media of ELISpot assay at 4th week of post-transplant could accurately discriminate acute rejection from stable graft function in kidney allograft recipients (Sensitivity: 83.3% and Specificity: 100.0%). Our results not only support previous reports on the role of IFN-γ-producing cells as predictive marker of acute rejection [5, 20, 22], but also help us to define a more reliable biomarker panel for kidney allograft recipients regarding their alloimmune status in early and even late post-transplant period. Remarkably, we showed a concurrent increase in proinflammatory cytokines (IFN-γ, IL-17 and IL-23) as prototypes for Th1 and Th17 cells along with decreased levels of TGF-β and low frequency of IL-10-producing cells in rejecting group of the patients. This may highlight the contribution of Th17 cells in allograft dysfunction in the presence of enhanced Th1 response in one hand and reinforce the protective role of IL-10 and TGF-β

Table 2
Univariate logistic regression analysis for the prediction of acute rejection by the frequencies of cytokine-producing cells and IL-23 levels.

Cytokine	B	SE	Wald	*p*-value	Odds Ratio	95% CI for OR	
						Lower	Upper
Interleukin-10	-0.091	0.029	10.051	0.002	0.913	0.862	0.966
Interleukin-17	0.181	0.049	13.521	< 0.001	1.199	1.088	1.321
Interferon-γ	0.079	0.025	9.710	0.002	1.083	1.030	1.138
Interleukin-23	0.058	0.030	3.657	0.056	1.060	.999	1.124

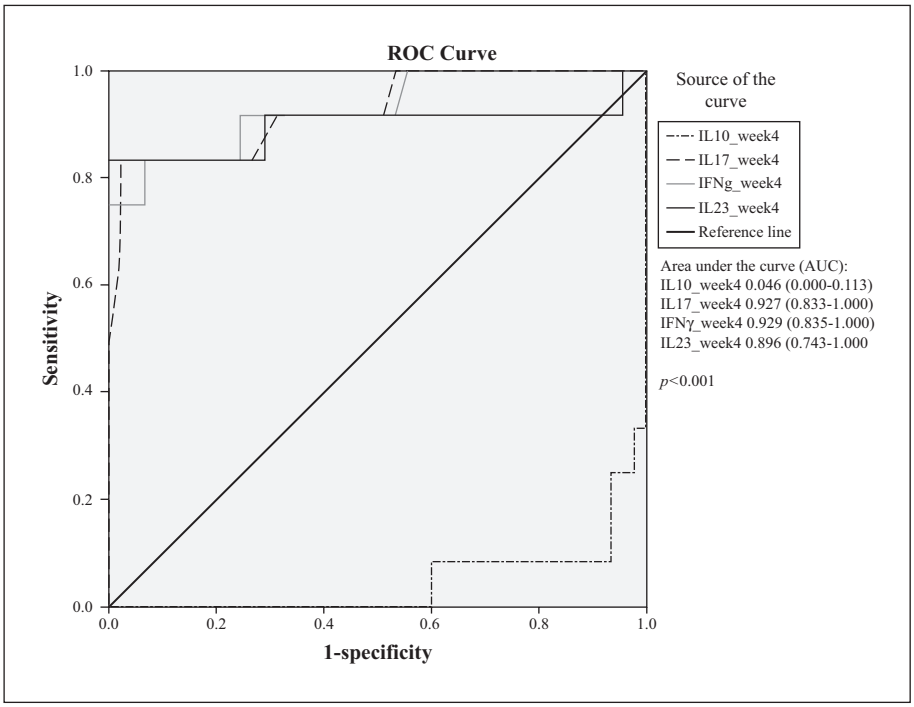


Figure 5
Receiver Operating Characteristic (ROC) analysis to determine the sensitivity and specificity of the frequencies of cytokine-producing cells for the prediction of acute rejection in kidney allograft recipients. AUC was 0.046 for IL-10 ($P < 0.001$), 0.927 for IL-17 ($P < 0.001$), 0.896 for IL-23 ($P < 0.001$) and 0.929 for INF-γ ($P < 0.001$) at week 4 post-transplant.

as prototypes of regulatory T cells for maintenance of graft stability on the other hand [13]. However, our randomized trial on kidney allograft recipients with low doses of donor bone marrow cells infusion clearly demonstrated that regulatory T-cell dominancy in the presence of high frequency of IL-10-producing cells and low frequencies of IL-17- and IFN-γ-producing cells was correlated with stable graft function both in early and late post-transplantation periods [23]. Nonetheless, our findings should be interpreted with caution and, definitely, further studies implementing larger cohort of patients and assessing more immune markers are warranted to determine the real predictive power of cellular immunity monitoring for early and late kidney allograft outcomes.

The proinflammatory role of Th17 cells in a number of autoimmune diseases or inflammatory conditions and limitedly in allograft rejection has been implicated. In this context, manipulation of the dynamic relationship between Th17 and regulatory T cells (Tregs) has been targeted to design therapeutic strategies to shift the Th17-Treg axis toward the stabilization of regulatory responses [12, 24, 25]. Our results in the current study reinforce interest for further investigations on Th17-Treg axis in transplantation rejection and tolerance, which, in turn, will provide crucial information for clinicians to determine whether and when a therapeutic intervention, either induction of regulatory T cells or additional immunosuppression, would be required.

However, although protocol/cause biopsies were not performed for all patients with clinical acute rejection episodes, a direct correlation between the increased frequencies of IFN-γ- and IL-17-producing donor-reactive cells in the rejecting group could be indicative for a donor-specific cellular immunity, which clearly discriminated patients at risk for early graft dysfunction. In contrast to some of the previous studies [5, 22], we did not observe significant differences for pretransplant frequencies of cytokine-producing cells as well as cytokine levels in the supernatants obtained during ELISpot assays between rejecting and stable graft function groups of the patients. This was probably due to recruitment of primary transplant patients without any possible prior sensitization. Of note, despite the small number of patients in the current study and relatively short follow-up period, our data convincingly showed that early post-transplant ELISpot monitoring of cellular immunity in kidney allograft recipients is a clinically feasible approach for categorizing renal transplant patients into low-risk and high-risk

Table 3
Prognostic criteria for acute rejection based on the frequencies of cytokine-producing cells and IL-23 level at 4th week post-transplantation.

Cytokine	Poor prognostic factors for SGF of 1-year graft survival
Interleukin-10	< 400 Spots/10^5 Cells (ELISPOT)
Interleukin-17	> 40 Spots/10^5 Cells (ELISPOT)
Interferon-γ	> 100 Spots/10^5 Cells (ELISPOT)
Interleukin-23	> 200 pg/mL (ELISA)

for early and potentially late immune-mediated graft failures.

In this regard, Crespo et al. [5] claimed that the first eight weeks post-transplantation is the most accurate time to detect T cell-mediated rejection (TCMR). They found a predictor of TCMR with IFN-γ ELISPOT assay, which had higher sensitivity and NPV (88.9% and 95.2%) but poorer specificity and PPV (62.5% and 40%). In line with these findings and more precisely, we observed that serial post-transplant ELISpot monitoring of IFN-γ-, IL-17- and IL-10-producing cells simultaneously could be strongly predictive for occurrence of acute rejection within the first month of post-transplantation and remarkably this predictive panel was shown to be more accurate at week 4 after transplantation. According to the significant differences between rejecting and SGF groups in terms of the frequencies of INF-γ-/ IL-10-/IL-17-producing cells, we could set a sensitive and specific cut-offs for the prediction of acute rejection within the first post-transplant year, which could be also feasible for the prediction of chronic rejection as well. As shown in *figure 1*, the earliest and best time for detecting these changes was the 4th week post-transplant because the differences between AR and SGF group were more prominent at this time point. Finally, we presented a valuable criterion for the early prediction of acute rejection before any clinical sign showed up and this, in turn, could help clinicians to perform in-time therapeutic interventions before the situation got worse. However, more studies with larger number of patients and longer follow-up are needed to prove the best criteria for the early diagnosis of either acute or chronic rejections. This would allow the tapering of immunosuppression in low-risk patients while those with high levels of alloreactivity might benefit from early additional immunosuppressive interventions.

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