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Brief communication

Serum PD-L1 and CTLA-4 levels as biomarkers of acute rejection and renal dysfunction in kidney transplant recipients

Angelica Canossi [a], Fabio Vistoli [b,c], Pierluigi Sebastiani [a], Alessia Colanardi [a], Tiziana Del Beato [a], Alessandra Panarese [b,c,*]

[a] Council National Research, Institute for Translational Pharmacology, Via Giosuè Carducci 32C – 67100, L'Aquila, Italy
[b] University of L'Aquila, Department of Biotechnological and Applied Clinical Sciences, Via Vetoio, Coppito 2 – 67100, L'Aquila, Italy
[c] San Salvatore Hospital ASL1 Abruzzo, Division of General and Transplant Surgery, Via Lorenzo Natali, Coppito – 67100, L'Aquila, Italy

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ABSTRACT

Purpose: Acute rejection in kidney transplantation is a critical barrier to long-term graft survival and the PD-1/PD-L1 and CTLA-4 molecules are crucial for the tolerance of alloreactive T cells against tubular epithelial cells. Our study aims to assess the role of these soluble PD-L1 and CTLA-4 molecules as biomarkers for *non-invasive* immunosurveillance after renal transplantation.

Methods: Blood samples from 65 recipients were investigated for serum sPD-L1 and sCTLA-4 molecules at the time of kidney transplantation (baseline), in 3 time-points (15, 60 and 365 days) post-transplant, and when acute rejection (ACR) was suspected. Samples and standards were processed in duplicate using sandwich ELISA.

Results: We revealed dynamic changes in serum expression over time, with a significant decrease from the time of kidney transplantation to the various monitoring points, except at the time of acute rejection when the levels increased. Multivariable logistic regression revealed that the sPD-L1 15-day post-transplant is an independent variable for ACR onset (AOR = 1.196 $p = 0.020$), and with a moderate discriminatory power (AUC = 0.717, $p = 0.031$), together with PD-L1 60 days, for the occurrence of rejection within 1 year from transplant. sPD-L1 after 15 days shows a predictive role also for DGF (AUC = 0.738, $p = 0.001$) and graft dysfunction at 60 days (AUC = 0.672, $p = 0.022$). Furthermore, a higher 15-day expression of sCTLA-4 in patients with ACR compared with those with stable graft (114.8 pg/mL vs. 67.8 pg/mL, $p = 0.018$) was reported.

Conclusion: These analyses suggest the potential role of these serum molecules as dynamic biomarkers of inflammation and immunoregulation in AKI and acute rejection; they may indicate new immunotherapy targets, useful for modulating tolerance and assist the clinician in identifying patients at risk of rejection or kidney failure.

1. Introduction

Acute rejection in kidney transplantation is a critical barrier to long-term graft survival, leading to chronic renal failure and progressive dysfunction [1]. Infiltrating alloreactive CD4+ and CD8+ cells primarily target proximal renal tubular cells, inducing inflammation and upregulating Major Histocompatibility Complex (MHC) class I and II molecules [2]. This facilitates T cell recognition and response. The interplay of co-stimulatory (B7-CD28) and co-inhibitory receptors (PD-1, CTLA-4) modulates T cell function. PD-1 interacts with its ligands, Programmed Cell Death-1 Ligand 1 (PD-L1) and (PD-L2), to transduce inhibitory signals that reduce T cell activity, proliferation and cytokine production [3]. Recent studies using viral infection models in mice have shown that rapid PD-1 expression on CD8+ T cells is induced by T cell

Abbreviations: Ab, Antibody; AKI, acute kidney injury; aTCMR, acute T-cell mediated rejection; CI, confidence interval; CTLA-4, Cytotoxic T-Lymphocyte-Associated Antigen 4; DGF, delayed graft function; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; ELISA, Enzyme-Linked Immunosorbent Assay; Eve, everolimus; FoxP3, forkhead box P3; IQR, interquartile ranges; IS, Immunosuppressive; MHC, Major Histocompatibility Complex; mTOR, mammalian target of rapamycin; OR, odds ratios; PD-1, Programmed Cell Death-1; PD-L1, Programmed Cell Death-1 Ligand 1; ROC, Receiver-operating characteristic; solCTLA-4, soluble Cytotoxic T-Lymphocyte-Associated Antigen 4; TCR, T cell receptor; TECs, tubular epithelial cells; Treg, regulatory T cell.

* Corresponding author at: University of L'Aquila, Department of Biotechnological and Applied Clinical Sciences, Via Vetoio, Coppito 2 – 67100, L'Aquila, Italy.
E-mail address: alessandra.panarese@univaq.it (A. Panarese).

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receptor (TCR) antigen signalling and maintained at high levels during expansion and granzyme B production [4]. PD-L1 reduces T-cell infiltration and promotes T exhaustion in both the spleen and transplanted organs. The choice of immunosuppressive therapy can influence PD-L1 ligand levels, as demonstrated in murine heart transplantation models, where tacrolimus upregulates PD-L1 expression in dendritic cells and allografts [5]. Clinical and experimental data implicate PD-L1 in graft rejection, with PD-L1 mRNA upregulation observed in renal allograft rejection biopsies, particularly in acute vascular rejection [6]. Upregulated PD-L1 on proximal tubular epithelial cells during acute allograft rejection may attenuate T-cell mediated injury [6]. Serum soluble PD-L1 (sPD-L1) levels vary between pathologies, and it is detected in bacterial and viral infetions, cancer patients, patients with autoimmune diseases and other pathologies and conditions including in pregnant women (Loacker et al., 2023). Iinflammatory diseases show lower levels compared to metastatic gastric cancer [7]. PD-L1 expression shows some variability due to the different biochemical/physiological forms of sPD-L1 and the heterogeneous cellular origin [8].

Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4) is another marker that has recently been investigated in kidney transplantation and cellular rejection. It plays a critical role in self-tolerance by acting as inhibitor co-receptor of CD4+ and CD8+ cellular responses and it is expressed primarily by regulatory T cells (Treg) [9–11]. CTLA-4 exerts its inhibitory function by various mechanisms, including the competitive action of its soluble form (solCTLA-4), which competes for CD28 binding and thereby attenuates T cell proliferation signals [12–14]. We have recently shown that solCTLA-4 transcripts detected at 15 days post-transplant may serve as predictors of increased risk of acute T-cell mediated rejection (aTCMR) over time and graft dysfunction at 1 year, and that solCTLA-4 pre-transplant levels have a protective effect against the development of de novo donor-specific antibodies (DSA) [15].

2. Objective

Our study aims to develop a *non-invasive* diagnostic tool in peripheral blood by monitoring these two markers in post-transplant period to predict acute kidney injury (AKI), acute rejection and graft dysfunction.

3. Materials and methods

3.1. Study population and recruitment process

This is a retrospective analysis performed on serum samples from kidney transplant recipients (KTR) prospectively collected in a single transplant center. We considered only patients with complete serum samples at the four-sample collection time-points and in case of acute rejection. All participants gave written informed consent, and the study adhered to the tenets of the Declaration of Helsinki, with approval from the institutional review board (prot.N.0098164/2011). Kidney recipients, previously examined at genetic level [15], underwent regular serum monitoring for PD-L1 and CTLA-4 molecular expression from pre-transplant time to 1-year post-transplant, at acute rejection and long-term. CTLA-4 expression was preliminarily assessed in a subset of 21 cases. Graft function was assessed by estimated glomerular filtration rate (eGFR), with chronic renal failure defined as eGFR$<$60 mL/min/1.73m^2 for $\geq$3 months [16]. The diagnosis of acute rejection was biopsy-proven according to Banff' 2022 classification [17]. KTRs were treated according to the local immunosuppression protocol. Induction therapy was carried out with basiliximab (Simulect®, Novartis, Basel, Switzerland) in 61 cases (92.4 %), and with antithymocyte globulins (Thymoglobulin®, Sanofi, Paris, France) in 5 cases (8.9 %). Maintenance therapy comprised prednisone, a calcineurin inhibitor (CNI) (either tacrolimus [Advagraf®,Astellas Pharma, Tokyo, Japan], in 56 cases (84.8 %), or cyclosporine [Neoral®, Novartis] in 10 cases (15.1 %), and a proliferation signal inhibitor. The latter consisted of mycophenolic acid in 55 cases (84.6 %), and everolimus (Certican®, Novartis) in 10 cases (15.54 %).

3.2. Sample collection and analysis protocols

Venous blood samples were collected at the time of kidney transplantation, in the post-transplantation period (15 days, 2 months and 12 months, long-term) and when rejection was diagnosed. Samples and standards were processed in duplicate using sandwich ELISA (Human Enzyme-Linked Immunosorbent assay kit) *(Human PD-L1 ELISA kit, Invitrogen; Human CTLA-4 SimpleStep ELISA kit, ABCAM*) to isolate relevant biomarkers, PD-L1 and CTLA-4. The optical density and analysis of PD-L1 and CTLA-4 concentrations (pg/mL) was assessed at 450 nm using ELx800 Absorbance Microplate Reader (BioTek instrument Inc.).

3.3. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 29.0 for Windows (SPSS Inc. Chicago, IL) and GraphPad Prism 6.0 (Boston, MA). Numerical variables were reported using means ± standard deviations (SD), or medians and interquartile ranges (IQR), as appropriate. Variability was estimated by calculating the coefficient of variation (CV). Results were compared using Fisher's exact test or Mann-Whitney *U* test/Wilcoxon. Comparison between groups and correlation between variables were examined by parametric (*t*-test/one-way ANOVA, Pearson's correlation), and non-parametric tests (Kruskal Wallis, Friedman test for repeated measures and Spearman's test). Significance criteria were set at $p \leq 0.05$. Logistic regressions were run for simply dichotomous variables. The crude odds ratios (OR), 95 % confidence interval (CI) and *p* value were reported for each predictor in the univariable analysis. Only statistically significant variables in the univariable analysis were entered into multiple logistic regression analysis to predict the final independent factors. Receiver-operating characteristic (ROC) curves were generated for the prediction of clinical events after transplantation to define the accuracy of diagnostic test and establish the best cut-off for clinical outcomes.

4. Results

Table 1 shows the clinical and demographical characteristics of the transplant recipients. Twenty-four patients (36.9 %) occurred a DGF. Fourteen patients showed at least one episode of rejection during the follow-up period, eleven cellular or mixed acute rejections (cellular and antibody-mediated rejections) and three showed chronic rejection episodes. The median time from transplantation to the first episode of aTCMR was 2.1 (IQR = 0.8–10.6) months in patients that experienced rejection within 1 year from transplantation. Fifteen recipients (23.1 %) developed de novo anti-DSA within the first year: all 15 (100.0 %) had DSA directed against HLA class I antigens and 7 (46.6 %) against class II.

4.1. PD-L1 and CTLA-4 serum expression patterns (Table 2)

PD-L1 expression patterns were monitored in peripheral blood samples in 65 kidney transplant recipients at various time points within one-year post-transplant and when possible long-term. The analysis revealed dynamic changes in PD-L1 expression levels over time, with a significant decrease from the time of transplantation (baseline time: median 21.44 pg/mL) to the various post-transplant monitoring points: 15 days (5.15 pg/mL, $p < 0.0001$), 60 days (4.32 pg/mL, $p < 0.0001$), up to 1 year (3.21 pg/mL) after kidney transplantation. In the long-term PD-L1 significantly rises compared to 1-year post-transplant (5.53 pg/mL), as well as at the time of rejection onset (6.52 pg/mL) **Supplementary Fig. 1.**

Preliminary assessment of CTLA-4 serum expression was conducted in a subset of 21 cases. We have encountered technical difficulties for this target in assaying many serum samples because of their low

Table 1
Demographic and clinical characteristics of the transplant study groups.

Variables	Frequencies, N (%)
Recipient gender:	
- males	47 (72.3)
- females	18 (27.7)
Recipient age (years, mean ± SD)	50.9 ± 13.2
Donor age (years, mean ± SD)	50.4 ± 15.7
Donor gender:	
- males	43 (66.2)
- females	22 (33.8)
Donor type:	
- brain-dead donors	61 (92.4)
- living donors	5 (7.6)
Time on RRT (months, mean ± SD)	54.9 ± 37.5
RRT type:	
- haemodialysis	53 (80.3)
- peritoneal dialysis	13 (19.7)
No of HLA mismatches (median and IQR)	3MM (2MM)
Class I PRA (%, mean ± SD)	4.9 ± 12.3
Class II PRA (%, mean ± SD)	2.2 ± 9.2
CIT (minutes, mean ± SD)	590 ± 248
WIT (minutes, mean ± SD)	44 ± 11
Induction:	
- basiliximab	61 (92.4)
- anti-thymocyte globulins	5 (7.6)
Calcineurin inhibitor:	
- cyclosporine	10 (15.1)
- tacrolimus	56 (84.8)
Proliferation signal inhibitor:	
- everolimus	10 (15.4)
- mycophenolic acid	55 (84.6)
CMV-positive donor/CMV-negative recipient	10 (15.1)
Previous transplantation	5 (7.6)
Delayed graft function	23 (35.4)

Table 2
PD-L1 and CTLA-4 molecular expression monitoring in kidney transplant recipients from pre-transplant time (baseline) to 1 year or long-term post-transplant period.

Biomarkers	n=	Mean (pg/mL)	Standard deviation (sd)	Coefficient of variation (%)
*PD-L1 baseline	65	21.40	7.872	36.79
PD-L1 15D	57	6.491	4.345	66.94
PD-L1 60D	56	5.631	4.057	72.04
PD-L1 1Y	14	3.271	1.725	52.72
PD-L1 LT	16	5.532	2.523	45.60
PD-L1 ACR	12	6.519	5.206	79.86
CTLA-4 baseline	21	113.20	164.000	144.85
CTLA-4 15D	15	86.60	46.470	53.66
CTLA-4 60D	19	79.01	36.220	45.84
CTLA-4 1Y	5	87.52	30.630	34.99
CTLA-4 LT	15	83.48	38.060	45.59
CTLA-4 ACR	5	98.130	65.230	66.48

15D = 15 days after transplantation, 60D = 60 days, 1Y = 1 year, LT = long time after tx, ACR = acute rejection. *1 way ANOVA Friedman test PD-L1 T0-T60 p < 0.000 (n = 39).

expression in healthy subjects. A decrease in the mean levels of CTLA-4 from baseline time (113.2 pg/mL) to the various time points in the post-transplantation period was recorded, but the mean values of CTLA-4 concentrations after transplantation remained quite constant. Only at the time of acute rejection a rise of expression was observed (98.13 pg/mL), however not significant.

Interestingly we can consider that a panel of 40 serum samples from randomly selected healthy donors tested for sPD-L1 expression by Invitrogen (Human PD-L1 ELISA kit) has a mean value of 26.5 pg/mL (range: 11.5–50.8 pg/mL). sCTLA-4 expression was absent or very low in healthy donors.

4.2. Effect of immunosuppressive (IS) therapy on PD-L1 expression

By monitoring PD-L1 levels in the post-transplant period according to the type of anti-proliferative Immunosuppressive (IS) drug (mycophenolate or everolimus), we reported a significantly higher production at 15 days in patients treated with everolimus (mean: 9.260 ± 6.650 vs. 5.702 ± 3.859 pg/mL, $p = 0.031$ 95 %CI = 0.344–6.771).

4.3. Biomarkers and the incidence of delayed graft function (DGF)

Examining the influence of these biomarkers on the DGF occurrence, we evidenced that sPD-L1 expression in patients who had experienced a DGF was significantly higher at 15 days post-transplant (7.07 vs. 4.692 pg/mL, $p = 0.0062$) **Supplementary Table 1**. No significant difference in the expression of CTLA-4 between the two groups of patients was observed instead. Logistic regression analysis also identified PD-L1 at 15 days as a correlated variable to this renal dysfunction ($p = 0.007$, Exp(B) = 1.298, 95 %CI = 1.076–1.567, B = 0.261). Analysis of the predictive model for DGF was performed using the receiver operating characteristic (ROC) curve obtained from PD-L1 protein levels measured 15 days post-transplant. Even though PD-L1 protein values were dosed later the event, the analysis showed a discriminatory power AUC = 0.738 with moderate accuracy for the occurrence of DGF (95 % CI = 0.595–0.881, $p = 0.001$). A cut-off >5.72 pg/mL was able to identify patients with higher risk of DGF with a sensitivity of 71.4 % and a specificity of 26.5 % (Youden's index of 0.450), **Supplementary Fig. 2.**

4.4. Biomarkers monitoring in relation to the occurrence of rejection episodes during the follow-up post-transplantation

Comparing the biomarker levels in the two groups of patients with rejection within 1 year ($n = 14$) and stable graft function ($n = 51$), we evidenced that sPD-L1 levels in patients with rejection were always higher in the various time points and progressively decreased after 60 days from transplantation (Table 3, **Supplementary Fig. 3 and Fig. 3**). Multivariable logistic regression revealed that the serum 15-day PD-L1 is an independent variable for acute rejection onset (AOR = 1.196 $p = 0.020$), Table 4.

To confirm the diagnostic accuracy of PD-L1 for identifying the risk of acute rejection, the receiver operating characteristic (ROC) analysis was performed by evaluating the area under the curve (AUC), considering serum PD-L1 levels measured 15 and 60 days after transplantation. The analysis showed a discriminatory power (AUC = 0.717) for PD-L1 15D and AUC = 0.650 for PD-L1 60D, with moderate accuracy for the

Table 3
PD-L1 and CTLA-4 protein expression in patients with or without acute T-cell mediated rejection (aTCMR).

Biomarkers	aTCMR (n = 14)		Stable graft (n = 51)		*p value=
	Mean (pg/mL)	Standard deviation	Mean (pg/mL)	Standard deviation	
PD-L1 Baseline	19.95	7.31	21.80	8.04	0.440
PD-L1 15D	8.18	6.50	6.04	3.53	0.542
PD-L1 60D	8.10	4.66	5.16	3.80	0.115
PD-L1 1Y	4.83	1.38	3.01	1.68	0.198
PD-L1 LT	3.82	3.81	5.78	2.39	0.516
PD-L1 ACR	6.30	5.92			
CTLA-4 Baseline	84.59	22.29	75.63	35.65	0.562
CTLA-4 15D	**114.80**	65.32	**65.79**	12.92	**0.026**
CTLA-4 60D	94.62	59.86	72.51	18.65	0.522
CTLA-4 1Y	65.53	12.64	95.25	29.19	0.266
CTLA-4 LT	68.68	6.89	90.71	45.46	0.516
CTLA-4 ACR	112.0	78.11			

* *Mann Whitney U test.*

Table 4
Univariable and multivariable logistic regression for the risk of acute rejection onset after kidney transplantation.

Variables	Univariable analysis			Multivariable analysis[a] -2log likelihood: 37.084		
	OR	95 % CI	P	AOR	95 % CI	P
Baseline			[a]			
CTLA4	1.010	0.980-1.042	0.512	–	–	–
PD-L1	0.965	0.891–1.045	0.383	–	–	–
At 15 days						
CTLA4	1.235	0.912–1.672	0.172	–	–	–
PD-L1	1.108	0.972–1.263	0.124	**1.196**	**1.029–1.390**	**0.020**
At 60 days						
CTLA4	1.017	0.984–1.051	0.309	–	–	–
PD-L1	1.137	0.979–1.320	0.092	–	–	–
At one year						
CTLA4	0.091		0.998	–	–	–
PD-L1	2.472	0.580–10.536	0.221	–	–	–
Longer time						
CTLA-4	0.929	0.724–1.191	0.559			
PD-L1	0.734	0.358–1.507	0.400			
Recipient age	1.019	0.969–1.019	0.460	–	–	–
Recipient gender	0.789	0.242–2.566	0.693	–	–	–
Donor age	1.010	0.975–1.046	0.579			
Dialysis time	1.001	0.989–1.001	0.889	–	–	–
CMV reactivation	1.230	0.449–3.371	0.688	–	–	–
CNI Tacrolimus vs. Cyclosporine	0.275	0.076–0.997	**0.049**	–	–	–
Use of everolimus	4.221	1.135–15.698	**0.032**	–	–	–
Immunosuppression change	3.889	1.212–12.481	**0.022**	–	–	–
DGF	2.130	0.711–6.388	0.177	–	–	–
Development of DSA	1.381	0.387–4.933	0.619	–	–	–
Proteinuria at one year (mg/l)	1.003	1.000–1.006	**0.039**			

[a] Model summary: $\chi^2(5) = 5.801$, $p = 0.016$; Nagelkerke $R^2 = 0.194$; Hosmer and Lemeshow χ^2 test = 6.705, $p = 0.458$. Covariates initially introduced in the multivariable model and then elided were: PD-L1 after 60 days from transplantation; everolimus, Maintenance immunosuppression change, proteinuria at 1 year post-transplant. Abbreviations: OR, odds ratio; CI, confidence intervals; AOR, adjusted odds ratio; DGF, delayed graft function.

occurrence of rejection within 1 year from transplant. A cut-off >5.092 pg/mL of PD-L1 15D has a higher risk of developing TCMR with a sensitivity of 87.5 % and a specificity of 43.6 % (Youden's index = 0.439), **Supplementary Fig. 4.**

Regarding the production of CTLA-4, we reported a significantly higher expression at 15 days post-transplant in patients with acute rejection, compared to that measured in patients with stable graft function (mean: 114.8 vs. 65.8 pg/mL, $p = 0.026$ U = 7.000). CTLA-4 expression at rejection onset was also high (112.0 pg/mL). In stable grafts, one-year levels increased, whereas they decreased in those with acute rejection onset (95.2 vs. 65.53 pg/mL), **Supplementary Fig. 5.** The reduced cases number available didn't permit to perform a diagnostic test for sCTLA-4 molecule.

4.5. Correlation analysis between experimental and clinical renal function data

In patients with acute rejection, correlation between molecular data and clinical renal function data showed that PD-L1 expression after 60 days was negatively associated with eGFR at 60 days ($p = 0.029$, $r = -0.685$) and positively associated with proteinuria at 1 year ($p = 0.047$, $r = 0.638$) and graft loss ($p = 0.026$, $r = 0.290$). PD-L1 expression at the acute rejection onset was also positively correlated with proteinuria at 15 days ($p = 0.002$, $r = 0.883$). In addition, PD-L1 baseline was positively correlated with 15-day eGFR ($p = 0.044$, $r = 0.256$) while 15-day PD-L1 levels had negative correlations with 15-day eGFR ($p = 0.011$, $r = -0.341$), 60-day eGFR ($p = 0.017$, $r = -0.321$) and 1-year eGFR ($p = 0.010$, $r = -0.348$). In patients with a stable graft, CTLA-4 expression at 15 days correlated positively with PD-L1 at 1 year ($p = 0.037$ $r = 0.900$). A negative correlation between PD-L1 levels of recipients pre-transplant and age exists ($p = 0.020$, $r = -0.241$).

4.6. Biomarkers and graft dysfunction

As regard the role of these molecules on 1-year graft dysfunction (expressed like eGFR<60 mL/min/1.73m^2), we observed always a higher serum PD-L1 levels in recipients with impaired graft function compared with those stable and significantly 1-year post-transplant ($p = 0.009$), . Univariable logistic regression showed a correlation between graft dysfunction after 1 year and serum PD-L1 expression at 1 year ($p = 0.037$ OR = 3.920), and at 60 days ($p = 0.044$, OR = 1.158), together with the covariates: recipient age ($p = 0.002$ OR = 1.071), donor age ($p < 0.0001$ OR = 1.094) and maintenance immunosuppression change ($p = 0.048$, OR = 3.397). No significant difference was observed for CTLA-4 expression. Besides, 15-days PD-L1 expression was correlated with graft dysfunction at 60 days ($p = 0.049$, exp.(B) = 1.204, 95 % CI = 1.001–1.447). PD-L1–15 days expression showed also a moderate discriminatory power AUC = 0.672 for the occurrence of graft dysfunction at 60 days after transplantation ($p = 0.022$) and a cut off

Table 5
Protein expression levels of PD-L1 and CTLA-4 in patients with or without graft dysfunction. (eGFR<60 ml/min/1.73m^2) or without (>60 ml /min/1.73m^2).

Biomarkers	eGFR < 60 ($n = 32$)		eGFR > 60 ($n = 28$)		
	Mean (pg/mL)	Standard deviation	Mean (pg/mL)	Standard deviation	*p value=
PD-L1 Baseline	22.31	6.62	20.89	9.16	0.489
PD-L1 15D	7.27	5.61	5.23	2.71	0.311
PD-L1 60D	6.26	5.16	3.90	2.66	0.209
PD-L1 1Y	4.79	2.47	1.72	1.37	**0.009**
CTLA-4 Baseline	141.3	226.2	90.81	32.18	0.724
CTLA-4 15D	80.86	34.19	103.5	72.22	0.999
CTLA-4 60D	72.12	13.98	95.93	55.00	0.757
CTLA-4 1Y	73.22	13.73	109.6	39.56	0.266

*Mann Whitney U test.

>5.204 pg/mL predicts a higher risk of low eGFR two months after transplant.

5. Discussion

Recent research indicates that elevated PD-L1 expression plays a key role in helping transplanted kidneys evade immune responses and establish immune tolerance [18]. Ex vivo experiments have demonstrated that PD-L1 contributes to T-cell apoptosis and suppresses their activation, leading to decreased local T-cell infiltration and enhanced differentiation of regulatory T cells (Tregs) [19]. Nevertheless, current treatments targeting PD-L1 or its receptor PD-1 remain clinically challenging, with limited data supporting their long-term effectiveness.

It's known that PD-L1 soluble form is cleaved from the membrane PD-L1 by matrix metalloproteinase and is increased by IFN-γ [20]. Soluble PD-L1 contributes to immune homeostasis together with membrane PD-L1 and exosome PD-L1. sPD-L1 is implicated in many diseases and conditions and its structural heterogeneity can lead to functional diversity. Besides, PD-L1 is correlated with a dual role with progression in renal diseases (lupus or glomerulonephritis) and this makes PD-L1 a nephrological marker [21]. sPD-L1 also binds to its receptor and is thought to be able to antagonize the inhibitory normal effect of mPD-L1 on T lymphocytes. [22].

Examining these preliminary data, we have observed a decrease in serum CTLA-4 and PD-L1 concentrations post-transplant in kidney recipients. Our data are consistent with recent findings obtained using flow cytometry analysis of Treg cells six months after transplantation. Peripheral blood Treg cell numbers are not stable, and they change from pre-transplant values throughout the evolution of the transplant, with a reduction of Treg cell levels after 6 months post-kidney transplantation from pre-transplant values [23,24]. However, interpreting PD-L1 and CTLA-4 levels in transplant recipients is complex due to various factors, including prior dialysis use and immunosuppressive drug effects, leading to expression variability. It is known that calcineurin inhibitors have a deleterious effect on Treg cells, whereas not mammalian target of rapamycin (mTOR) and that there is an inverse correlation between the circulating Treg cells and tacrolimus blood levels.

Comparison of kidney transplant recipients based on acute rejection outcomes showed always higher PD-L1 expression in rejection cases compared to those with stable graft, up to one year follow-up, and an association with sPD-L1 levels after 15 days post-transplant obtained from a multivariable regression. Previous studies have linked elevated soluble PD-L1 levels to impaired renal function, also in absence of infection, compared with those with normal renal function [25].

These results indicate that high levels of serum soluble PD-L1 molecule may be influenced by impaired graft function and that the PD-L1 soluble form in this biological context is more a marker of an inflammatory status of endothelial cells to regulate T regulatory expansion than just an immunosuppressive marker in regulating and maintaining peripheral tolerance []. It could act inhibiting the uppression of T cell activation, interfering with PD-L1/PD-1-mediated immunosuppression. Manipulating of the PD-L1/PD-1 pathway post-transplantation may help this mechanism of allospecific Treg expansion.

In this study the sPD-L1 results to be an independent risk factor for rejection occurrence and its 15D levels with a good predictive ability for acute rejection onset (AUC = 0.717) within the first year from transplant. sPD-L1 expression levels stay higher after kidney transplantation also in patients with graft dysfunction or DGF and PD-L1 levels 15 days post-transplant show a good predictive power to identify high-risk recipients.

In addition, previous studies had linked elevated sPD-L1 to impaired renal function, also in absence of infection, compared with those with normal renal function [25]. All these results reflect also the evidence that their release is dependent by cell activation or exhaustion status of graft immunity.

On the other hand, sCTLA-4 levels remain almost constant in among pre- and post-transplant period in recipients with stable transplantation but instead they significantly rise 15-day post-transplant in acute rejection cases. Our previous genetic studies support the role of soluble CTLA-4 in rejection onset [15,26]. Later after 1 year and long-term from transplantation, CTLA-4 levels increase in stable transplants while they decrease in recipients with acute rejection. A possible explanation could be that both CTLA-4 isoforms (membrane and soluble) play an important role in establishing and maintaining peripheral tolerance [27]. Although initial observations indicated that solCTLA-4 is predominantly produced by resting T cells [28], subsequent studies have demonstrated that its release increases during antigenic responses [29]. This association between day-15 solCTLA-4 and acute rejection might originate from the influence of Tregs on memory CD8 + CD28 − Teff, which has been recently implied in allograft dysfunction [30]. It is known that end-stage renal disease (ESRD) patients harbour a heterogeneous population of CD3 + CD8 + CD28− cells with immunomodulatory but also cytotoxic characteristics to a greater extent than healthy subjects do, and which expands after transplantation. We noted that despite the relatively small number of acute rejection cases and, overall, the small sample size of the entire cohort should represent a note of caution for any definitive interpretation, we hypothesize a putative role of early post-transplant levels of solCTLA-4 on the susceptibility to acute rejection, through an influence on T-cell activation.

Regarding the impact of immunosuppressants on the expression of the two biomarkers CTLA-4 and PD-L1, our findings indicate that everolimus (Eve) may influence PD-L1 expression, in the early post-transplantation period, especially 15-days post-transplant, potentially impacting transplant outcomes. It is established that inhibitors of the mammalian target of rapamycin (mTOR), like Eve, exhibit not only immunosuppressive effects through the inhibition of T-cells, but also antiproliferative, anti-chemotactic, and anti-neo angiogenetic effects. PD-L1 was found to be significantly elevated in tumour endothelial cells isolated from everolimus-treated tumours. Combining mTOR inhibitors with immune checkpoint inhibitors could enhance antitumor efficacy, reducing rejection risk.

Such data should be also interpreted considering the interplay of CTLA-4 and PD-L1 pathways which control immune response: both the molecules compete for CD80 binding and CTLA-4 can regulate PD-L1: PD-1 interaction via trans-endocytosis of CD80. The interaction between CD80 and PD-L1 may preclude PD-1 binding, preventing PD-1-mediated inhibition [31].

6. Limitations

The principal limitations of this study are the relatively small sample size and the heterogeneity of the biochemical and physiological forms of PD-L1, not distinguishable using the ELISA technique. The possibility of an influence of different PD-L1 isoforms in results was noticed but the analysis aims only to identify these molecules in peripheral blood to verify their possible use like biomarkers of inflammation or immune dysregulation after kidney transplantation in specific events. The goal would be to consider these serum molecules as a drug target.

The prospects are to expand the case history of patients enrolled considering validation in a larger independent cohort. In addition, we aim to investigate the interplay between PD-L1 ligand and its PD-1 receptor, together with CTLA-4 responsible for the inhibitory pathway of the immune response, to give clearer indications on renal damage (AKI) of high-risk patients and outcome with ICI treatment.

7. Conclusion

This longitudinal study of serum PD-L1 and CTLA-4 expression in the peripheral blood of KTRs, before and after transplantation, suggests that these molecules may have a crucial role in the immunosurveillance after renal transplantation. Despite the known immunosuppressive function of PD-L1 and CTLA-4, *serum* biomarkers (soluble or other molecular

forms) increase in inflammatory status and graft injury in post-transplant period. An in-depth study of the function of these markers in transplantation may indicate new immunotherapy targets useful for modulating tolerance and assist the clinician in identifying patients at risk of rejection or kidney failure by non-invasive procedures, thus enabling the predictive medicine and development of personalised immunosuppression protocols and/or ICI treatments in kidney transplant patients.

CRediT authorship contribution statement

Angelica Canossi: Data curation, Conceptualization, Writing – review & editing, Writing – original draft. **Fabio Vistoli:** Supervision, Resources, Conceptualization, Writing – review & editing. **Pierluigi Sebastiani:** Methodology, Investigation. **Alessia Colanardi:** Methodology, Investigation. **Tiziana Del Beato:** Methodology, Investigation. **Alessandra Panarese:** Validation, Supervision, Resources, Data curation, Conceptualization, Writing – review & editing.

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Declaration of competing interest

A statement on ethics approval is included in Methods. Written informed consent was obtained from all subjects and the project was approved by the local ethics committee of ASL n.1 (protocol n. 0098164/2011). Blood samples were provided by the Unit of General Surgery and Transplantation of San Salvatore Hospital in L'Aquila, Italy. Consent for storage and further use of biological materials up to 10 years after the end of the trial is included as optional part of the informed consent.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trim.2025.102250.

Data availability

Data will be made available on request.

References

[1] H.U. Meier-Kriesche, A.O. Ojo, J.A. Hanson, D.M. Cibrik, J.D. Punch, A. B. Leichtman, et al., Increased impact of acute rejection on chronic allograft failure in recent era, Transplantation 70 (7) (2000) 1098–1100, https://doi.org/10.1097/00007890-200010150-00018.

[2] L.D. Cornell, R.N. Smith, R.B. Colvin, Kidney transplantation: mechanisms of rejection and acceptance, Annu. Rev. Pathol. 3 (2008) 189–220, https://doi.org/10.1146/annurev.pathmechdis.3. 121806.151508.

[3] G.J. Freeman, A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, et al., Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation, J. Exp. Med. 192 (7) (2000) 1027–1034, https://doi.org/10.1084/jem.192.7.1027.

[4] E. Ahn, K. Araki, M. Hashimoto, et al., Role of PD-1 during effector CD8 T cell differentiation, Proc. Natl. Acad. Sci. USA 115 (18) (2018) 4749–4754, https://doi.org/10.1073/pnas.1718217115.

[5] X. Luo, G. Du, B. Chen, G. Yan, L. Zhu, P. Cui, et al., Novel immunosuppressive effect of FK506 by upregulation of PD-L1 via FKBP51 in heart transplantation, Scand. J. Immunol. 96 (4) (2022) e13203, https://doi.org/10.1111/sji.13203.

[6] A. Starke, M.T. Lindenmeyer, S. Segerer, M.A. Neusser, B. Rüsi, D.M. Schmid, et al., Renal tubular PD-L1 (CD274) suppresses alloreactive human T-cell responses, Kidney Int. 78 (1) (2010) 38–47, https://doi.org/10.1038/ki.2010.97.

[7] Z. Zheng, Z. Bu, X. Liu, L. Zhang, Z. Li, A. Wu, et al., Level of circulating PD-L1 expression in patients with advanced gastric cancer and its clinical implications, Chin. J. Cancer Res. 26 (1) (2014) 104–111, https://doi.org/10.3978/j.issn.1000-9604.2014.02.08.

[8] C. Bailly, X. Thuru, B. Quesnel, Soluble programmed death Ligand-1 (sPD-L1): a Pool of circulating proteins implicated in health and diseases, Cancers (Basel) 13 (12) (2021) 3034, https://doi.org/10.3390/cancers13123034.

[9] T. Takahashi, T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, et al., Immunologic self-tolerance maintained by CD25(+) CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4, J. Exp. Med. 192 (2) (2000) 303–310, https://doi.org/10.1084/jem.192.2.303.

[10] L.S. Walker, D.M. Sansom, The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses, Nat. Rev. Immunol. 11 (12) (2011) 852–863, https://doi.org/10.1038/nri3108.

[11] K. Wing, T. Yamaguchi, S. Sakaguchi, Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation, Trends Immunol. 32 (9) (2011) 428–433, https://doi.org/10.1016/j.it.2011.06.002.

[12] O.S. Qureshi, Y. Zheng, K. Nakamura, K. Attridge, C. Manzotti, E.M. Schmidt, et al., Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4, Science 332 (6029) (2011) 600–603, https://doi.org/10.1126/science.1202947.

[13] U. Grohmann, C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, et al., CTLA-4-Ig regulates tryptophan catabolism in vivo, Nat. Immunol. 3 (11) (2002) 1097–1101, https://doi.org/10.1038/ni846.

[14] F. Fallarino, U. Grohmann, K.W. Hwang, C. Orabona, C. Vacca, R. Bianchi, et al., Modulation of tryptophan catabolism by regulatory T cells, Nat. Immunol. 4 (12) (2003) 1206–1212, https://doi.org/10.1038/ni1003.

[15] A. Canossi, S. Iesari, Q. Lai, S. Ciavatta, T. Del Beato, A. Panarese, et al., Longitudinal monitoring of mRNA levels of regulatory T cell biomarkers by using non-invasive strategies to predict outcome in renal transplantation, BMC Nephrol. 23 (1) (2022) 51, https://doi.org/10.1186/s12882-021-02608-3.

[16] A.S. Levey, L.A. Stevens, C.H. Schmid, Y.L. Zhang, A.F. Castro 3rd, Feldman HI et al a new equation to estimate glomerular filtration rate, Ann. Intern. Med. 150 (9) (2009) 604–612.

[17] M. Naesens, C. Roufosse, M. Haas, C. Lefaucheur, R.B. Mannon, B.A. Adam, et al., The Banff 2022 kidney meeting report: reappraisal of microvascular inflammation and the role of biopsy-based transcript diagnostics, Am. J. Transplant. 24 (3) (2024 Mar) 338–349, https://doi.org/10.1016/j.ajt.2023.10.016.

[18] J. Yang, L.V. Riella, S. Chock, T. Liu, X. Zhao, X. Yuan, et al., The novel costimulatory programmed death ligand 1/B7.1 pathway is functional in inhibiting alloimmune responses *in vivo*, J. Immunol. 187 (2011) 1113–1119, https://doi.org/10.4049/jimmunol.1100056.

[19] M. Ben Nasr, S. Tezza, F. D'Addio, C. Mameli, V. Usuelli, A. Maestroni, et al., PD-L1 genetic overexpression or pharmacological restoration in hematopoietic stem and progenitor cells reverses autoimmune diabetes, Sci. Transl. Med. 9 (416) (2017), https://doi.org/10.1126/scitranslmed.aam7543 eaam7543.

[20] M.P. Pistillo, R. Carosio, B. Banelli, A. Morabito, L. Mastracci, P. Ferro, et al., IFN-γ upregulates membranous and soluble PD-L1 in mesothelioma cells: potential implications for the clinical response to PD-1/PD-L1 blockade, Cell. Mol. Immunol. 17 (4) (2020) 410–411, https://doi.org/10.1038/s41423-019-0245-x.

[21] Y. Wei, Z. Jiang, The role of programed death-ligand 1 in renal diseases, J. Recept. Signal Transduct. Res. 40 (4) (2020) 295–300, https://doi.org/10.1080/10799893.2020.1734820.

[22] K.W. Ng, J. Attig, G.R. Young, E. Ottina, S.I. Papamichos, I. Kotsianidis, G. Kassiotis, Soluble PD-L1 generated by endogenous retroelement exaptation is a receptor antagonist, Elife 8 (2019) e50256, https://doi.org/10.7554/eLife.50256.

[23] C. Caprara, G.R. Kinsey, V. Corradi, W. Xin, J.Z. Ma, E. Scalzotto, et al., The influence of hemodialysis on T regulatory cells: a Meta-analysis and systematic review, Blood Purif. 42 (4) (2016) 307–313, https://doi.org/10.1159/000449242.

[24] D. San Segundo, L.H. Galván-Espinoza, E. Rodrigo, J. Irure, et al., Regulatory T-cell number in peripheral blood at 1 year Posttransplant as predictor of Long-term kidney graft survival, Transplant. Direct 5 (3) (2019 Feb 8) e426, https://doi.org/10.1097/TXD.0000000000000871.

[25] L. Loacker, A. Egger, V. Fux, R. Bellmann-Weiler, G. Weiss, A. Griesmacher, et al., Serum sPD-L1 levels are elevated in patients with viral diseases, bacterial sepsis or in patients with impaired renal function compared to healthy blood donors, Clin. Chem. Lab. Med. 61 (12) (2023) 2248–2255, https://doi.org/10.1515/cclm-2023-0232.

[26] A. Canossi, A. Aureli, F. Delreno, S. Iesari, C. Cervelli, K. Clemente, et al., Influence of cytotoxic T-lymphocyte antigen-4 polymorphisms on acute rejection onset of cadaveric renal transplants, Transplant. Proc. 45 (7) (2013) 2645–2649, https://doi.org/10.1016/j.transproceed. 2013.07.008.

[27] L.N. Dahal, H. Schwarz, F.J. Ward, Hiding in plain sight: soluble immunomodulatory receptors, Trends Immunol. 39 (10) (2018) 771–774, https://doi.org/10.1016/j.it.2018.08.004.

[28] G. Magistrelli, P. Jeannin, N. Herbault, et al., A soluble form of CTLA-4 generated by alternative splicing is expressed by non-stimulated human T cells, Eur. J. Immunol. 29 (11) (1999 Nov) 3596–3602, https://doi.org/10.1002/(SICI)1521-4141(199911)29:11<3596::AID-IMMU3596>3.0.CO;2-Y.

[29] F.J. Ward, L.N. Dahal, S.K. Wijesekera, et al., The soluble isoform of CTLA-4 as a regulator of T-cell responses, Eur. J. Immunol. 43 (5) (2013) 1274–1285, https://doi.org/10.1002/eji.201242529.

[30] M. Strioga, V. Pasukoniene, D. Characiejus, CD8+ CD28- and CD8+ CD57+ T cells and their role in health and disease, Immunology 134 (1) (2011) 17–32, https://doi.org/10.1111/j.1365-2567.2011.03470.x.

[31] M.A. Robinson, A. Kennedy, C.T. Orozco, H.C. Chen, E. Waters, D. Giovacchini, et al., Rigid, bivalent CTLA-4 binding to CD80 is required to disrupt the cis CD80/PD-L1 interaction, Cell Rep. 43 (9) (2024 Sep 24) 114768, https://doi.org/10.1016/j.celrep.2024.114768.35.