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CD16+ Cells and Costimulatory Molecules of Lymphocyte Activation Present inside Human Kidney Grafts and in Blood Circulation

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Abstract

Background: We studied the expression of important costimulatory molecules of lymphocyte activation and the presence of CD16+ cells on aspiration biopsies of kidney transplants, measured three soluble factors and when indicated tested their robustness in diagnosing acute rejection. Methods: Fine-needle aspiration biopsies were performed either on days seven or 14 - 30 post-transplantation among stable kidney transplants and on the day of acute rejection diagnosis, while a sample of peripheral blood was collected simultaneously. The cyto preparations were studied by the enzymatic avidin biotin complex staining. The immunocytochemistry was directed to CD16, CD28, CD152, ICOS, CD40, CD154, CD26 and CD27. We performed the analysis in the peripheral blood by ELISA for soluble(s) CD16, CD26, and CD154. Results: The group of acute rejection cases showed a significant up-regulated expression of CD16, CD26, ICOS and CD40 as compared to the group of stable cases. Both sCD16 and sCD154 were significantly higher in the blood samples of the group with acute rejection. Thymoglobulin down-regulated CD154 and sCD16. CD16, CD26 and ICOS exhibited very high sensitivity and specificity for acute rejection diagnosis. Conclusions: The presence of CD16+ cells inside the graft emerged as a distinct player in acute rejection, confirming other previous reports whereas we first document that in human kidney transplants, ICOS and CD26 are significantly up-regulated and both reached positive predictive values for acute rejection ≥ 80%. The other costimulatory molecules, with the exception of CD40, though widely known, did not show robust association with immune events.

Keywords

Kidney Transplants, Aspiration Biopsies, Costimulatory Molecules, CD16+

Cells

1. Introduction

Organ transplantation walked a very long and exciting road. Coming from the first laboratory experiments which raised deep reservation for its feasibility [1] currently, human kidney transplants (KTx) enjoy excellent short-term outcomes although less than optimal long-term survival [2]. The etiologies of KTx failures are diverse and have quite different frequencies depending on the time interval from the surgical procedure but immune-mediated injury occupies a central role [2] [3], notwithstanding the significant betterments of immunosuppressor drugs developed during the last four decades. Rejection of KTx, or of any other organ transplant, demands an elaborate cooperation between different cells and their products. In order to kindle the rejection process, antigen must be processed and presented to a lymphocyte but this step is not able *de per se* to start the reaction and a second step of cell activation is needed, both on the antigen-presenting cell side and on the responder cell side [4], to reach maximal efficacy and escape from anergy. Several molecules which perform this costimulatory function have been identified [5] and some of them have already raised the opportunity to investigate the clinical efficacy of drugs interfering with their actions [6] [7] [8]. The functional importance is not the same for these costimulatory molecules and, not unexpectedly, some controversy remains about descriptions of their full effects, how immunosuppressors affect them as well as their associations with clinical events in human transplantation [5] [9] [10] [11].

Different antigen-presenting cells have been identified with different abilities for T cell priming [12] and although previous studies have highlighted that the expression of CD16 is associated with better antigen presenting ability [13], others have claimed quite the contrary [14].

Per se fine-needle aspiration biopsy (Fnab) in kidney transplants, described by P Haÿry [15] has not arrived at a desirable level of diagnostic accuracy but when the aspiration samples are further analysed by flow cytometry, they provide the answer as to whether the KTx is developing an acute rejection or not, with very high reliability [16]. Of importance, when compared with peripheral blood, Fnab samples display significant differences for several T cell subsets [17] raising doubts over whether studies of peripheral blood can be a reliable mirror of what is happening inside the graft, although it may be possible to identify markers at the peripheral blood level correlated with clinical status, and monitoring or even prediction of events.

We took advantage from our Fnab program in KTx to get an easy and ethically acceptable gateway into intra-graft immune events. We selected a group of molecules to study in human KTx, including three members belonging to the immunoglobulin superfamily, CD28, CD152 and ICOS, and two of the

TNF-TNF-receptor superfamily, CD27, CD154. We added the study of CD40, the pair ligand for CD154. We also included in our study another costimulatory molecule, CD26, which is preferentially expressed on activated $CD4^+$ lymphocytes and memory T cells [18]. We analysed the presence of $CD16^+$ cells in Fnab samples completing our antecedent study of $CD14^+$ cells [19]. Lastly, we measured soluble(s) CD16, CD26 and CD154 in peripheral blood samples.

2. Patients and Methods

This study included 129 adult KTx, aged twenty to sixty-five years. Each patient provided adequate Fnab samples according to the criteria defined by P Haÿry [15] and all received an organ from a deceased donor. The study group was not of consecutive KTx, it included the cases where Fnab was possible for logistic reasons and when sample proved to be of quality following P Haÿry [15]; the acute rejection frequency of KTx in the transplant unit averages 15% of cases). Table 1 summarizes patient's demographics and characteristics.

Table 1. Demographics and characteristics. Values are reported in the given units and all comparisons between groups were nonsignificant, except serum creatinine that was higher in recipients with AR ($p = 0.002$). Recipients are all adult, aged between 20 - 65 years old and transplanted with kidney deceased donors. AR diagnosis were done on the biopty-gun biopsy, done at the same time as Fnab, and classified according to Banff criteria and secured by a positive response to treatment or by histologic reassessment of graft nephrectomy. DN-diabetes mellitus, IgA-IgA nephropathy, RPGN-rapidly progressive glomerulonephritis, SLE-systemic lupus erythematous, FSGS-focal segmental glomerulosclerosis, TIN-tubulointerstitial nephritis, HTN-hypertension, CGN-chronic glomerulonephritis, PKD-adult polycystic kidney disease, DGF-delayed graft function.

Phenotype characteristic	All Ktx recipients (n = 129)	Stable recipients (n = 92)	Rejection recipients (n = 37)
Gender			
Female	52	38	14
Male	77	54	23
Cause of ESRD			
DN	24	18	6
IgA	14	10	4
RPGN	9	4	5
SLE	5	3	2
FSGS	8	7	1
TIN	35	25	10
HTN	6	4	2
CGN	20	15	5
PKD	8	6	2
Immunosupression			
CsA	98	71	27

Continued

RAPA	7	6	1
MMF	25	15	10
Anti-IL2αR	7	7	0
Ktx			
First	121	87	34
Re-Ktx	8	5	3
DGF	23	15	8
Serum Creatinine (mg/dL)	NA	2.8	4.9

Each patient was treated from the outset with a calcineurin inhibitor, plus mycophenolate mofetil (MMF) and prednisolone, with the exception of the second grafts that received quadruple sequential therapy, including two to five doses of thymoglobulin, according to the number of blood lymphocytes; also, in seven first KTx rapamycin (RAPA) substituted for MMF. The therapeutic target whole blood levels for CsA, TAC and RAPA during the first three months post-KTx were 150 - 250 ηg/ml, 6 - 12 ηg/ml and 4 - 12 ηg/ml, respectively. All had a panel of reactive antibodies less than 10% with the exception of second transplants.

Ninety-two KTx remained rejection-free for the first year post-KTx, at least, 71 treated with CsA and 21 with TAC, including six cases treated with RAPA. Thirty-seven KTx developed an acute rejection episode at a median of 15.0 ± 430 days post-KTx, 22 of them occurred during the first month, five cases during the second and third month, and ten cases after the third month post-KTx. Twenty seven of these acute rejection cases were treated with CsA and ten with TAC, including one patient treated with RAPA and three with quadruple therapy. Every acute rejection episode was diagnosed by a biopty-gun biopsy done at the same time as the Fnab procedure and read by an independent pathologist following the standardized Banff criteria. The rejection diagnosis was further secured by either a positive response to treatment or by histologic reassessment of graft nephrectomy. Acute rejection was treated with either 1) 3 pulses of IV 250 - 500 mg methylprednisolone, 2) thymoglobulin, minimum of two doses, or 3) 5 - 12 sessions of plasmapheresis and IgG at 0.4 gr/kg weight, if the episode was graded IIa or greater or when c4d positivity was observed along with donor-specific antibodies. Only one case was proved to be treatment resistant, and this patient suffered a transplantectomy at the third week post-KTx.

All patients received prophylaxis with ganciclovir/valganciclovir when the donor was positive and the recipient was negative for CMV, and whenever thymoglobulin was administered. Furthermore, each KTx received cotrimoxazol as prophylaxis for Pnemocystis jirovecii during the first six to twelve months post-surgery.

Among the rejection-free cases, Fnab was done on day seven and on days 14

or 30 post-KTx, 90 - 150 min after the morning intake of immunosuppressive drugs, and on the day of the biopty-gun biopsy among rejection cases. The corresponding blood sample was drawn with the Fnab procedure. As a rule, one patient provided one sample for a costimulatory molecule. Fnab samples were submitted to a 700 rpm cytocentrifugation for 10 min and kept at −70˚C until testing.

2.1. Immunocytochemistry Studies

Cytoslides were brought back to room temperature and they were submitted to the immunocytochemistry studies by the enzymatic Avidin Biotin Complex (ABC) method using the detection system UltraVision™, HRP/DAB (Horseradish Peroxidase/Three, 3'Diaminobenzidine Tetrahydrochloride) from Thermo Scientific, UK. All the incubations were done at room temperature. Briefly, cytoslides were hydrated in ethanol 95˚ and incubated with hydrogen peroxide for 15 min to peroxidase blocking, followed by a rinse in distilled water and Tris Buffered Saline (TBS) solution at pH = 7.4. Then, for unspecific immunoglobulin blocking, a 10 min incubation with Ultra V Block, from Lab Vision™. After removal of the excess of the unspecific serum, the primary antibody at the appropriated dilution was added for a 60 min incubation. In the end, the cytoslides were washed in distilled water and dipped for 10 min in TBS before a 10 min incubation with the secondary antibody, at a concentration of 4 μg/ml both if goat anti-mouse or rabbit anti-goat. After rinse of the secondary antibody excess with TBS a new incubation for 10 min with Streptavidin Peroxidase, followed with 10 min rinse in TBS and then the addition of DAB Chromogen and DAB Substrate for 10 min incubation. Finally, hematoxylin from Mayer's Hematoxylin, DakoCytomation was applied followed by a rinse in running tap water for 2 min and one min dehydration with ethanol 95˚, and a coverslip with Entellan® mounting medium. The primary antibodies used included a mouse IgG_{1k} (clone 3-G8) at a 35 μg/ml, for CD16, a mouse IgG_{1k} (clone M-A261) at 25 μg/ml for CD26, a mouse IgG_{1k} (clone M-T271) at 20 μg/ml for CD27, a mouse IgG_{1k} (clone CD28.2) at 20 μg/ml for CD28, a mouse IgG_{1k} (clone M5C3) at 20 μg/ml for CD40, a mouse $IgG_{2a,k}$ (clone BNI3) at 20 μg/ml for CD152, a mouse IgG_{1k} (clone RAP1) at 20 μg/ml for CD154, all from Pharmingen™ and a goat IgG at 10 μg/ml directed to ICOS from R & D Systems. From each cytoslide every kidney tubular cell (R) was counted as well as every lymphocyte and monocyte-macrophage (L/M), both negative and positive for the antibody in order to present the absolute values of positive cells as well as the ratio of positive cells for both R cells and for L-M cells in an attempt to correct for the variation in Fnab sample contents in cells.

2.2. ELISA Studies in Sera Samples

The analysis of soluble CD16 in serum was done by ELISA according with the manufacturer instruction, Ray Bio®, using microtiter plate wells coated with pu-

rified human FcγRIII/CD16 antibody, where each 10 µl sample was diluted 40 µl sample diluent. For evaluation of soluble CD26, we used an ELISA kit from RayBio® applying 100 µl of sample. The analysis for soluble CD154 in serum was done by ELISA from R & D Systems, 100 µl sample was used.

2.3. Statistical Analysis

The statistical analysis included the determination of median, SD, and interquartile ranges for the costimulatory molecules. The comparisons for serum creatinine and whole blood immunosuppressor drugs were done by unpaired Student's T test, and the comparative analysis for costimulatory molecules results both by immunocytochemistry and ELISA was done by Mann-Whitney U test. The correlations between costimulatory molecules were tested using Spearman correlation. The sensitivity, specificity, negative predictive value (NPV) and positive predictive (PPV) and areas under the ROC were evaluated when indicated.

This study was approved by the Faculty of Medicine of University of Porto and University Hospitalar Center of S. João, Porto joint Committee of Ethics. Informed consent was obtained from each patient and the study was conducted in compliance with the Good Clinical Practice Guidelines declaration of Helsinki and Istanbul.

3. Results

The median and interquartile range for serum creatinine among rejection-free cases on day seven post-KTx was 2.8 mg/dl and 1.6 - 7 mg/dl, respectively and 4.9 mg/dl and 2.6 - 10 mg/dl, respectively for acute rejection group ($P = 0.002$), 2.5 mg/dl and 1.6 - 10 mg/dl on days 14 and 30 post-KTx combined ($P > 0.05$ on comparing day seven with day 14 - 30 combined stable cases, $P = 0.009$ when comparing day 14 and 30 combined with acute rejection day). Fifteen cases of delayed graft function were remarked among rejection-free cases and eight among acute rejection cases. The whole blood levels for CsA, TAC and RAPA were within the limits of the transplant unit protocol in almost every case and no episode of calcineurin toxicity or of clinical important CMV disease occurred. However, both CsA and TAC blood levels were significantly inferior among acute rejection as compared with all the rejection-free KTX, $P < 0.000$ and $P = 0.005$, respectively. No significant difference was observed when comparing HLA matching for rejection-free cases with acute rejection cases but a significant correlation was observed between the presence of anti-HLA antibodies pre-KTx and acute rejection ($P = 0.002$).

In Table 2 and Table 3, we present the results for CD16 and CD26 expression in Fnab cells. No significant correlation was observed between both $CD16^+$ and $CD26^+$ with either serum creatinine or blood drug levels. Also, no difference was observed for both molecules whether or not RAPA was present in the drug treatment. On comparing stable cases with acute rejection ones, the wider difference was observed for the absolute values of positive cells for both molecules. The statistical analysis and diagnostic performance are shown in Table 4, highlighting the good predictive values, both for $CD16^+$, PPV > 71.4% and $CD26^+$,

Table 2. CD16 expression in Fnab cells. Group A, stable KTx; A1, Fnab on day 7; A2, Fnab on days 14 - 30 days post-KTX; Group B, acute rejection group. Results expressed as absolute number of positive cells, ratio of positive over renal parenchymal cells (R), ratio of positive over total of lymphocyte-monocyte/macrophage cells (LM); values given as median ± SD and lower and upper quartiles in brackets.

CD16	A1 *n = 24*	A2 *n = 14*	B *n = 10*	Mann-Whitney *p*
Absolute number	22.3 ± 47.6 [0 - 14]	45.8 ± 106.3 [1 - 46]	292.8 ± 383.8 [27 - 534]	*A1 vs A2: 0.51* ***A vs B: 0.0002***
+cells/Rcells	0.04 ± 0.089 [0 - 0.03]	0.07 ± 0.09 [0.01 - 0.13]	0.53 ± 0.7 [0.05 - 0.83]	*A1 vs A2: 0.41* ***A vs B: 0.0017***
+cells/LM	0.054 ± 0.086 [0 - 0.055]	0.1 ± 0.13 [0.1 - 0.014]	0.67 ± 0.88 [0.11 - 0.8]	*A1 vs A2: 0.15* ***A vs B: 0.002***

Table 3. CD26 expression in Fnab cells. Group A, stable KTx; A1, Fnab on day 7; A2, Fnab on days 14-30 days post-KTX; Group B, acute rejection group. Results expressed as absolute number of positive cells, ratio of positive over R cells, ratio of positive over total of LM cells; values given as median ± SD and lower and upper quartiles in brackets.

CD26	A1 *n = 16*	A2 *n = 6*	B *n = 11*	Mann-Whitney *p*
Absolute number	12.9 ± 23.3 [0 - 13.5]	19.8 ± 26.2 [8 - 17]	69.9 ± 54.2 [32 - 130]	*A1 vs A2: 0.238* ***A vs B: 0.0003***
+cells/Rcells	0.052 ± 0.085 [0 - 0.096]	0.041 ± 0.034 [0.02 - 0.067]	0.294 ± 0.476 [0.044 - 0.336]	*A1 vs A2: 0.48* ***A vs B: 0.003***
+cells/LM	0.045 ± 0.083 [0 - 0.049]	0.045 ± 0.037 [0.015 - 0.08]	0.177 ± 0.144 [0.053 - 0.313]	*A1 vs A2: 0.28* ***A vs B: 0.001***

PPV 80%, when taking into account the absolute values of positive cells, cut-off 35 and 33, respectively. (NPV was 93.5% and 95.8% in the same order). Figure 1 and Figure 2 show the results assessed for the AUC of the ROC curves and the distribution of stable patients and acute rejection group, either for absolute number of positive cells, ratio of positive cells over R cells or ratio of positive cells over LM cells, for CD16 and CD26 respectively. Among eight stable KTx, both molecules were simultaneously studied, and the correlation was 0.67, P = 0.07.

We analysed sera samples for sCD16 and sCD26 in a group of 37 stable KTx, day 7 post-surgery and 18 acute rejection cases developed during the first month post-KTx. sCD16 was significantly higher in acute rejection, P = 0.014, and the serum values were significantly lower among thymoglobulin cases as compared to non-thymoglobulin, P = 0.008. For sCD26, we did not observe significant differences either comparing acute rejection cases with stable cases or among different drug treatments.

In Table 5 and Table 6, we present the results for CD28 and CD152, respectively. No significant differences were observed for $CD28^+$. As for $CD152^+$ within the stable KTx, we found a trend to down-regulation in day 14/30 combined versus day 7 post-surgery, which reached a significant value for the ratio of $CD152^+$/total L/M.

Table 4. Diagnostic performance for $CD16^+$, $CD26^+$ and $ICOS^+$. The AUC for the ROC curve, the sensitivity and specificity reached for cut-off points for absolute values, ratio of positive over renal cells (R), ratio of positive over total lymphocytes plus monocytes (LM).

	AUC	Sensitivity	Specificity	Cut-off
CD16				
Absolute number	0.914	0.83	0.88	27.5
+cells/Rcells	0.876	0.83	0.82	0.04
+cells/LM cells	0.876	0.67	0.97	0.21
CD26				
Absolute number	0.973	0.89	0.96	32
+cells/Rcells	0.929	1.0	0.80	0.04
+cells/LM cells	0.942	0.89	0.88	0.09
ICOS				
Absolute number	0.937	0.93	0.81	5.5
+cells/Rcells	0.926	0.93	0.74	0.02
+cells/LM cells	0.939	1.0	0.81	0.01

Table 5. CD28 expression in Fnab cells. Group A, stable KTx; A1, Fnab on days 7 and 14; A2, Rapamycin, day 7 post-KTX; Group B, acute rejection group. Results expressed as absolute number of positive cells, ratio of positive over R cells, ratio of positive over total of LM cells; values given as median ± SD and lower and upper quartiles in brackets.

CD28	A1 *n = 17*	A2 *n = 6*	B *n = 11*	Mann-Whitney ***p***
Absolute number	10.6 ± 15.6 [0 - 13]	19.6 ± 34.4 [1.5 - 22]	9.54 ± 23.7 [0 - 8]	*A1 vs A2: 0.31* *A vs B: 0.70*
+cells/Rcells	0.032 ± 0.056 [0 - 0.03]	0.063 ± 0.092 [0.003 - 0.099]	0.027 ± 0.66 [0 - 0.115]	*A1 vs A2: 0.21* *A vs B: 0.79*
+cells/LM	0.031 ± 0.049 [0 - 0.032]	0.06 ± 0.097 [0.008 - 0.055]	0.045 ± 0.124 [0 - 0.022]	*A1 vs A2: 0.16* *A vs B: 0.70*

Table 6. CD152 expression in Fnab cells. Group A, stable KTx; A1, Fnab on day 7; A2, stable, Fnab on days 14 - 30 post-KTX; Group B, acute rejection group. Results expressed as absolute number of positive cells, ratio of positive over R cells, ratio of positive over total of LM cells; values given as median ± SD and lower and upper quartiles in brackets.

CD152	A1 *n = 19*	A2 *n = 7*	B *n = 9*	Mann-Whitney ***p***
Absolute number	22.2 ± 24.9 [1 - 46]	10.9 ± 18.9 [0 - 29]	17.6 ± 24.1 [2 - 19]	*A1 vs A2: 0.088* *A vs B: 0.88*
+cells/Rcells	0.65 ± 0.1 [0.003 - 0.057]	0.024 ± 0.047 [0 - 0.042]	0.06 ± 0.1 [0.007 - 0.037]	*A1 vs A2: 0.08* *A vs B: 0.82*
+cells/LM	0.098 ± 0.11 [0.008 - 0.204]	0.017 ± 0.029 [0 - 0.042]	0.043 ± 0.063 [0.005 - 0.034]	*A1 vs A2:* ***0.034*** *A vs B: 0.49*

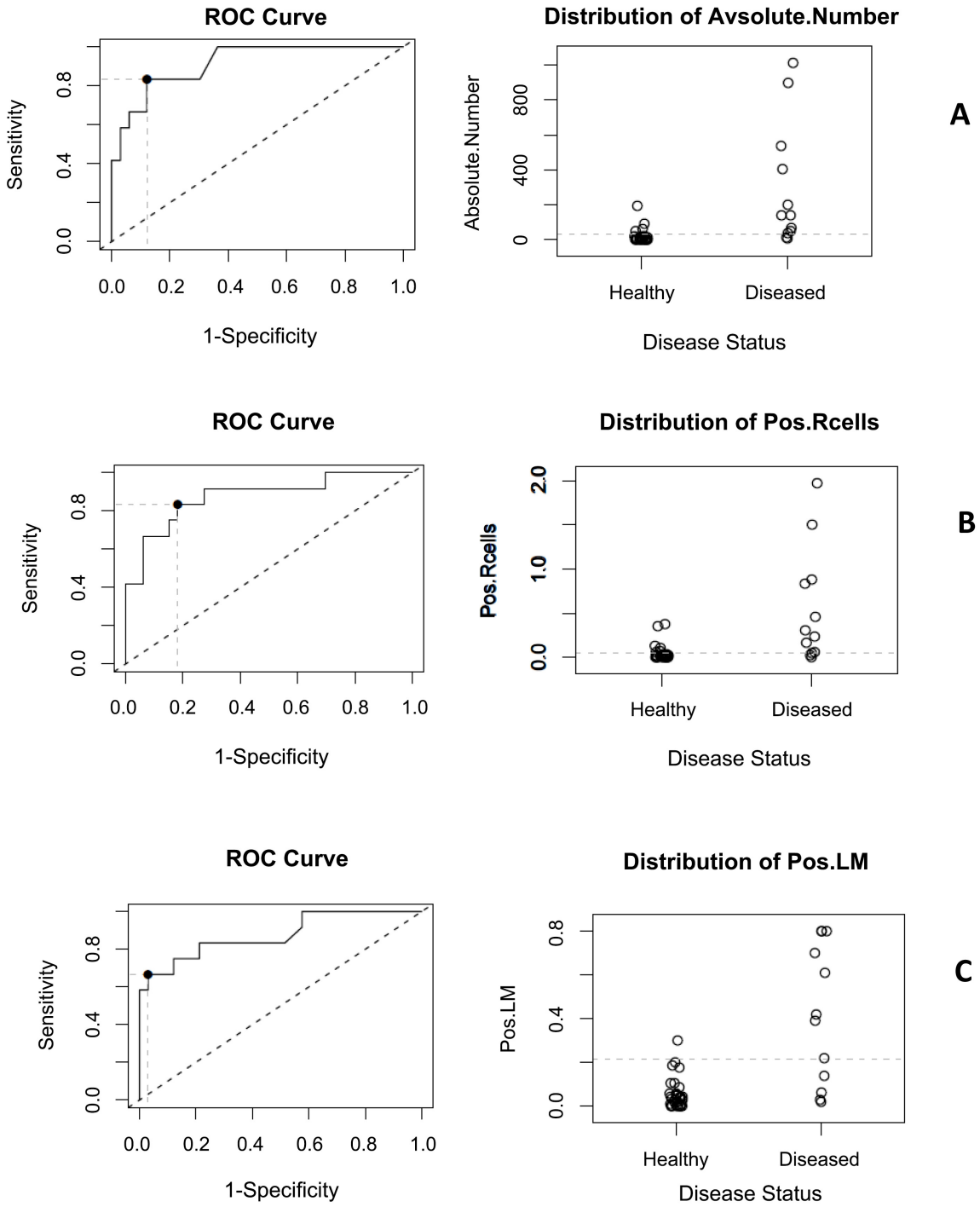

Figure 1. CD16 costimulatory molecules segregate nonrejection (NR) patients from those with acute rejectin (AR). The score model was practiced on38 NR and 10AR samples to generate a scale Q score ranging from 0 to 800for Absolute Number (AN) and from 0 to 2 for Pos.Rcells and from 0 to 0.8 for Pos.LM ratios. The AUC of the ROC curves and the disribution of NR and AR are shown in the figure. (A) For AN the Youden optimal cut-off method set a threshold at 35 with a corresponding sensitivity of 83.3% and specificity of 87.9%. The AUC of the ROC curve was 0.914 ($p < 0.000$). (B) For the ratio PosCelR the Youden optimal cut-off method set a threshold at 0.05 with a corresponding sensitivity of 83.3% and specificity of 81.8%. The AUC of the ROC curve was 0.876 ($p < 0.000$). (C) For the ratio PosLM the Youden optimal cut-off method set a threshold at 0.218 with a corresponding sensitivity of 66.7% and specificity of 97%. The AUC of the ROC curve was 0.876 ($p < 0.000$).

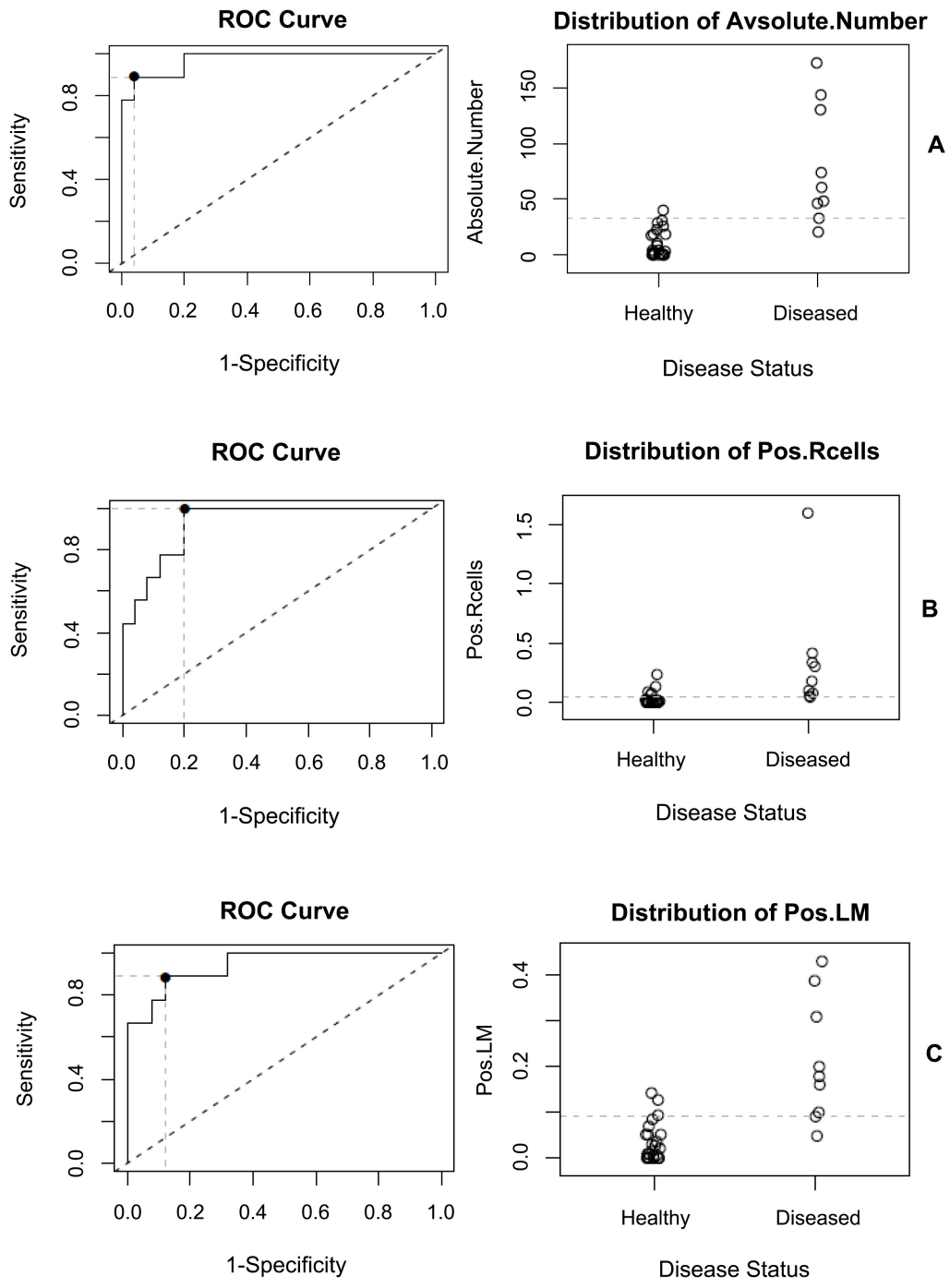

Figure 2. CD26 costimulatory molecules segregate nonrejection (NR) patients from those with acute rejectin (AR). The score model was practiced on 22 NR and 11 AR samples to generate a scale Q score ranging from 0 to 150 for Absolute Number (AN) and from 0 to 1.5 for Pos.Rcells and from 0 to 0.4 for Pos.LM ratios. The AUC of the ROC curves and the distribution of NR and AR are shown in the figure. (A) For AN the Youden optimal cut-off method set a threshold at 33 with a corresponding sensitivity of 88.9% and specificity of 96.0%. The AUC of the ROC curve was 0.937 ($p < 0.000$). (B) For the ratio PosCelR the Youden optimal cut-off method set a threshold at 0.044 with a corresponding sensitivity of 100% and specificity of 80%. The AUC of the ROC curve was 0.929 ($p < 0.000$). (C) For the ratio PosLM the Youden optimal cut-off method set a threshold at 0.09 with a corresponding sensitivity of 88.9% and specificity of 88%. The AUC of the ROC curve was 0.942 ($p < 0.000$).

For ICOS, the analysis is shown in Table 7. Again the diagnostic performance was robust independent of considering the absolute values of $ICOS^+$, the ratio with renal cells and the proportion of $ICOS^+$ of the total of lymphocytes plus monocytes present in the Fnab sample, reaching PPV values equal or above 80% and NPV higher than 85%, Table 4 and Figure 3.

In Table 8, we present the results for CD27 and in Table 9 and Table 10 we present the quantifications for CD154 and CD40, respectively. There were a significantly higher number of $CD40^+$ cells among the rejection KTx but the difference turned to be non-significant when analysing the ratios over R or LM. A significant down-regulation of $CD154^+$ was found among the stables treated with thymoglobulin as compared to no thymoglobulin, P = 0.026. A significant correlation was found between $CD40^+$ with $CD154^+$, r = 0.57, P = 0.012 and quite unexpectedly, between $CD154^+$ with $CD152^+$, r = 0.80, P = 0.001.

The quantification of soluble CD154 in the serum showed a significantly higher level among rejection versus stable cases, P < 0.000. Defining a cut-off value for acute rejection diagnosis ≥ 50 pg/ml, PPV was 63.6% and NPV 85.9%. The correlation between the expression of CD154 in Fnab samples and soluble CD154 was both non-significant and negative, r = −0.17.

Table 7. ICOS expression in Fnab cells. Group A, stable KTx, days 7 and 14 combined; Group B, acute rejection group. Results expressed as absolute number of positive cells, ratio of positive over R cells, ratio of positive over total of LM cells; values given as median ± SD and lower and upper quartiles in brackets.

ICOS	A *n = 31*	B *n = 23*	Mann-Whitney *p*
Absolute number	6.71 ± 9.6 [0 - 8]	31.6 ± 55.9 [3 - 30]	***A vs B: 0.0063***
+cells/Rcells	0.025 ± 0.048 [0 - 0.034]	0.122 ± 0.32 [0.009 - 0.096]	***A vs B: 0.011***
+cells/LM	0.028 ± 0.059 [0 - 0.02]	0.096 ± 0.161 [0.014 - 0.1]	***A vs B: 0.0013***

Table 8. CD27 expression in Fnab cells. Group A, stable KTx, A1, Fnab on day 7; A2, Fnab on days 14 - 30 post-KTX; Group B, acute rejection group. Results expressed as absolute number of positive cells, ratio of positive over R cells, ratio of positive over total of LM cells; values given as median ± SD and lower and upper quartiles in brackets.

CD27	A1 *n = 14*	A2 *n = 8*	B *n = 10*	Mann-Whitney *p*
Absolute number	15.6 ± 16.7 [2 - 21]	16 ± 19.3 [4 - 19]	32.5 ± 40.5 [0 - 55]	*A1 vs A2: 0.70* *A vs B: 0.88*
+cells/Rcells	0.068 ± 0.088 [0.011 - 0.088]	0.063 ± 0.076 [0.007 - 0.107]	0.063 ± 0.084 [0 - 0.123]	*A1 vs A2: 0.68* *A vs B: 0.83*
+cells/LM	0.058 ± 0.070 [0.011 - 0.084]	0.065 ± 0.054 [0.022 - 0.126]	0.67 ± 0.105 [0 - 0.111]	*A1 vs A2: 0.63* *A vs B: 0.43*

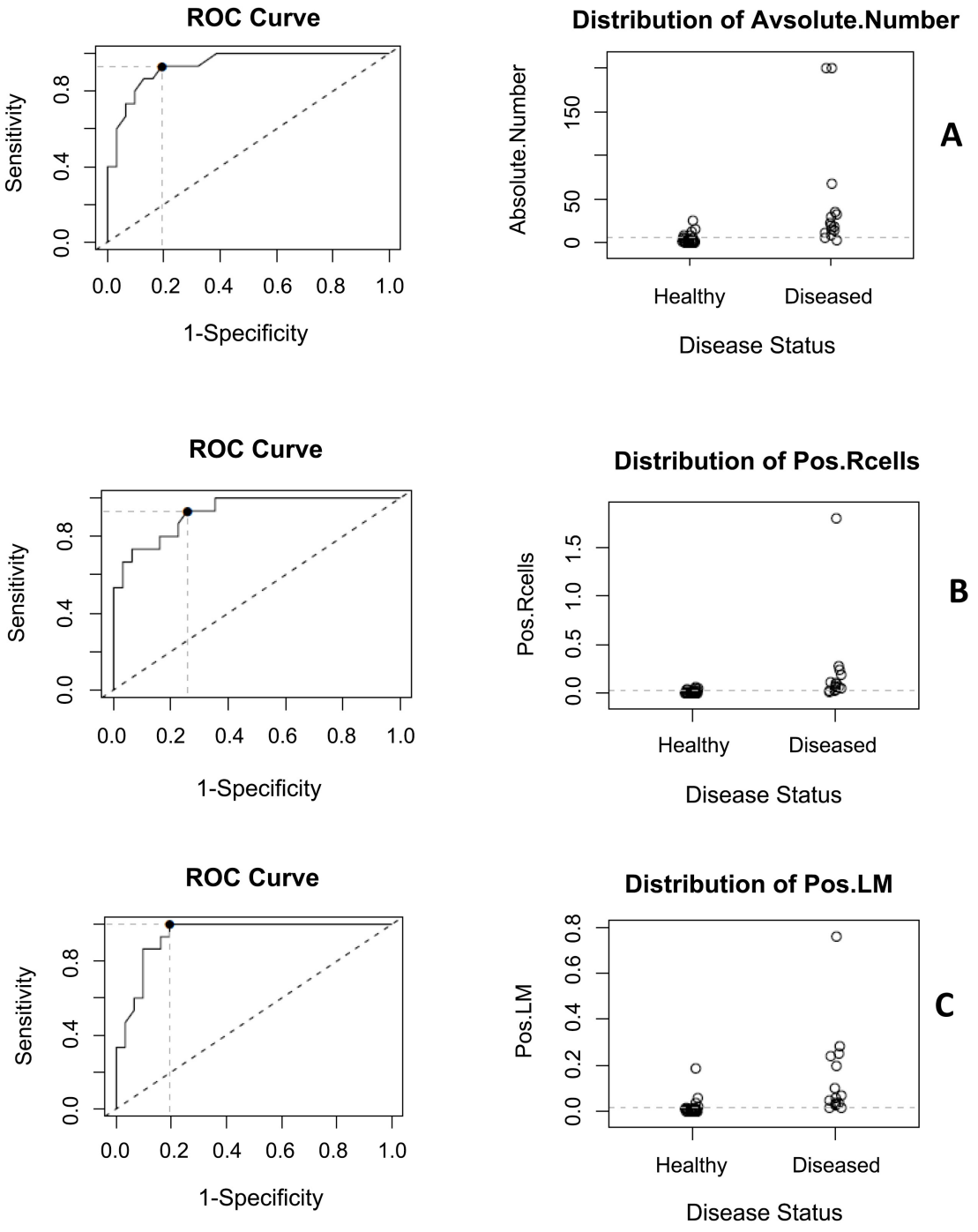

Figure 3. ICOS costimulatory molecules segregate nonrejection (NR) patients from those with acute rejectin (AR). The score model was practiced on 31 NR and 23 AR samples to generate a scale Q score ranging from 0 to 150 for Absolute Number (AN)and from 0 to 1.5 for Pos.Rcells and from 0 to 0.8 for Pos.LM ratios. The AUC of the ROC curves and the disribution of NR and AR are shown in the figure. (A) For AN the Youden optimal cut-off method set a threshold at 6 with a corresponding sensitivity of 93.3% and specificity of 80.6%. The AUC of the ROC curve was 0.937 ($p < 0.000$). (B) For the ratio PosCelR the Youden optimal cut-off method set a threshold at 0.02 with a corresponding sensitivity of 93.3% and specificity of 74.2%. The AUC of the ROC curve was 0.926 ($p < 0.000$). (C) For the ratio PosLM the Youden optimal cut-off method set a threshold at 0.014 with a corresponding sensitivity of 100% and specificity of 80.6%. The AUC of the ROC curve was 0.939 ($p < 0.000$).

Table 9. CD154 expression in Fnab cells. Group A, stable KTx, A1, stable, Fnab on day 7; A2, stable, Fnab on days 14 - 30 post-KTX; Group B, acute rejection group. Results expressed as absolute number of positive cells, ratio of positive over R cells, ratio of positive over total of LM cells; values given as median ± SD and lower and upper quartiles in brackets.

CD154	A1 *n = 18*	A2 *n = 10*	B *n = 9*	Mann-Whitney *p*
Absolute number	21.1 ± 29.2 [1 - 24]	74 ± 121.9 [1 - 81]	45.2 ± 51.9 [8 - 73]	*A1 vs A2: 0.53* *A vs B: 0.52*
+cells/Rcells	0.058 ± 0.074 [0.01 - 0.084]	0.386 ± 0.811 [0.001 - 0.09]	0.087 ± 0.127 [0.006 - 0.107]	*A1 vs A2: 0.96* *A vs B: 0.81*
+cells/LM	0.1 ± 0.11 [0.01 - 0.163]	0.287 ± 0.515 [0.002 - 0.188]	0.153 ± 0.235 [0.031 - 0.114]	*A1 vs A2: 0.84* *A vs B: 0.91*

Table 10. CD40 expression in Fnab cells. Group A, stable KTx, days 7 and 14 combined; Group B, acute rejection group. Results are expressed as absolute number of positive cells, ratio of positive over R cells, ratio of positive over total LM cells; values given as median ± SD and lower and upper quartiles in brackets.

CD40	A *n = 26*	B *n = 12*	Mann-Whitney *p*
Absolute number	8.38 ± 14.4 [0 - 11]	28.6 ± 46.9 [6.5 - 18]	***A vs B: 0.042***
+cells/Rcells	0.027 ± 0.055 [0 - 0.02]	0.17 ± 0.42 [0.007 - 0.098]	*A vs B: 0.086*
+cells/LM	0.045 ± 0.069 [0 - 0.062]	0.092 ± 0.157 [0.005 - 0.07]	*A vs B: 0.37*

4. Discussion

Our study shows a significantly higher presence of $CD16^+$, $CD26^+$ and $ICOS^+$ cells, and a trend to significantly higher expression of $CD40^+$ among Fnab samples obtained from a group of acute rejection KTx. This rejection group included different types of rejection but only one case was treatment-resistant and, in agreement with others, a predominance of mixed rejection with both a cellular and humoral components was observed [20]. Previously, we also reported in Fnab samples that $CD14^+$ cells were counted at significantly higher numbers among steroid-resistant but not among steroid-sensitive cases [19]. That study showed a ratio of renal cells/$CD14^+$ cases in steroid-sensitive and steroid-resistant cases equal to 1 ± 5.5 and 0.6 ± 1.0, respectively which is of similar magnitude with close to two $CD16^+$ per renal cells, now (**Table 2**). Notwithstanding with different treatment protocols, we have not seen relevant influences of different immunosuppressors on CD16 expression in opposition to what was observed at peripheral blood of KTx by Neudoerfl *et al.* [21]. Actually, the association between monocytes and $CD16^+$ cells has already been observed by others [22] [23] but their diagnostic performance has not been reported, which we have done and just by looking at the absolute values of $CD16^+$ cells present in Fnab samples

we observed a PPV higher than 70% and an NPV 93.5%, when the incidence of acute rejection in our unit has varied between 12% to 18% cases along the last decade. These predictive values are lower but of the same order of magnitude of what we showed for lymphocyte subset analysis by flow cytometry in Fnab samples [16] [17], slightly and indirectly suggesting that both lymphocytes and $CD16^+$ cells play a role in anti-graft response with similar importance even when looking at the same time post-transplant.

CD16 expression is quite wide-range. It is known to be expressed in granulocytes, natural killer cells, monocytes and macrophages, and activated endothelial cells [24]. The most widely quoted CD16 functions include the facilitation of antibody-dependent cellular cytotoxicity and lysis of some virus-infected cells and tumor cells [25]. The involvement of $CD16^+$ natural killer cells in the acute rejection of KTx is known [26] and CD16 gene polymorphisms are associated with strength and frequency of rejection in KTx [27] and lung transplants [28]. However, the $CD16^+$ cells present in our Fnab samples were for the most part monocytes-macrophages; natural killer cells were easily identified and were not commonly seen. $CD16^+$ monocytes represent up to 15% of peripheral blood monocytes and they highly express HLA-DR antigens which may underlie its better antigen-presenting capacity [29] while they are endowed with the highest ability to promote allogeneic T cell proliferation [30]. $CD16^+$ monocytes become migratory dendritic cells in a model tissue setting [31]. We surmise that $CD16^+$ cells observed in Fnab samples are mainly monocytes in a differentiation process towards dendritic cells starting the assembly of the anti-graft response in acute rejection cases. This is translated by the ratio of $CD16^+$/R cells increasing tenfold, such as it is observed in stable KTx and as shown in **Table 2** and in agreement with H. Wu suggestion in the very elegant report of single-cell transcriptomics of human KTx biopsies [32].

CD26 study almost replicates the measurements of CD16, the ratio of positive cells over renal cells increased fivefold as compared to what was observed in stable KTx, as shown in **Table 3**. Again, the diagnostic performance of $CD26^+$ in Fnab was quite robust reaching 80% for PPV and an NPV above 95%. Of interest, among a group of eight stable KTx cases studied simultaneously for CD16 and CD26, we found a significant positive correlation. Of note, no significant correlation was observed between sCD26 and the clinical status of KTx, contrary to sCD16 where we observed a significantly higher level in rejection cases. As far as we are aware, our report is the first to show a strongly significant association of $CD26^+$ cells infiltrating KTx and acute rejection in humans.

CD26 expression is widespread including several types of immunocompetent cells but also endothelial and epithelial cells [5]. This enzyme regulates several physiological processes, namely T cell activation, lymphocyte-epithelial cell adhesion [5], and its expression is inhibited by calcineurin inhibitors, RAPA and MMF [33]. Specific inhibitors of CD26 suppress T cell proliferation *in vitro* and decrease antibody production in a mice model [34], and prolongs allograft survival in a rat heart transplant model [35]. Also, in a rat syngeneic lung transplant

model, CD26 enzymatic activity inhibition was followed by a decrease in ischemia/reperfusion injury [36]. In CD26 knockout mice, skin allotransplants showed a delay in graft rejection with decreased synthesis of pro-inflammatory cytokines, enhancement of IL-10 production and of T regulatory cells [37].

The pair CD28-CD152 did not show differences when comparing with acutely rejecting KTx. While CD28 pivotal role in facilitating alloimmune responses is agreed [38], rejection develops unfettered in MHC mismatched mice recipients genetically deficient in CD28 [39]. The use of selective CD28 blockers in non-human primate models revealed better grafting as compared to belatacept probably by preserving the action of CTLA-4 on regulatory T cells, among other effects [40]. On the other hand, certain T cell subsets, namely CD8 memory cells are less dependent on signalling through this pathway [41]. Of interest, the report of CD28 down-regulation by mycophenolic acid [42] was not observed in our study as no difference was found when comparing RAPA which substituted for MMF with non-RAPA.

We believe that our findings with CD28 might be confounded by its constitutive expression on naïve and activated T cells [5].

As for CD152, and unexpectedly, we only observed a significantly lower expression on immunocompetent cells on day 14 as compared to day 7 post-surgery in stable KTx. CD152 is up-regulated on activated $CD4^+$ and $CD8^+$ T cells and outcompetes with CD28 for CD80 and CD86 ligation, blocking the CD28 positive signalling [43]. Perhaps our results were influenced by the rapid internalization of surface CD152 which is 80% accomplished within five minutes [44].

We observed a strong association of $ICOS^+$ with KTx acute rejection. Actually, the ratio of $ICOS^+$ cells over R displayed the higher PPV for acute rejection (84.6%) among this group of costimulatory molecules, jumping fourfold in rejection as compared to stable KTx. ICOS is not expressed on resting T cells but is rapidly up-regulated upon T cell activation [45]. However, ICOS overexpression on donor-reactive T cells is unable to enhance alloreactive CD8 T cell responses or precipitate acute rejection in a mouse model of skin allotransplant [46]. Also, a delayed ICOS-Ig failed to enhance survival of heart transplants in cynomolgus monkey [47]. Perhaps, in human KTx, ICOS may turn to be more important as suggested by the finding of more abundant $ICOS^+$ graft-infiltrating cells accompanied by higher plasma ICOS levels in antibody mediated rejection [48]. Previously, E Akalin reported a significant association of $ICOS^+$ cells in human KTx developing chronic glomerulopathy [49] but patients enduring acute rejection were not included, and thus it seems that our study is the second study dealing with $ICOS^+$ cells in human studies following that of E Akalin. Of interest, a study in nonhuman primate KTx using an ICOS-Ig human Fc-fusion protein failed to find any result although the molecule used was not able to prevent the graft infiltration by $ICOS^+$ cells, too [50]. Again, taking into account the diagnostic performance of graft-infiltrating $ICOS^+$, we believe it is reasonable to advance that in human KTx under current immunosuppressive ICOS signalling, plays a significant and non-redundant role in the anti-allograft response.

CD40 analysis showed a significant up-regulation in acute rejection and a close to significantly higher value for the ratio of $CD40^+$ over R, this ratio increasing six fold as compared to what was measured in stable KTx. On the contrary, $CD154^+$ in Fnab displayed no differences, only a significant difference comparing stable with rejection when looking at sCD154. Not unexpectedly, $CD40^+$ correlated positively and significantly with $CD154^+$ in Fnab within a small group where both were tested in the same sample. The relevance of CD40/CD154 pair is firmly established both for the development of thymus-dependent humoral immune response and in T-cell mediated dendritic cell activation/maturation and monocyte-macrophage activation [51] [52] [53]. Trials realized in non-human primate models anticipate the entrance of CD40 and or CD154 antagonists in the near future for human Tx [54].

The role CD27 plays in transplantation is not defined. It is expressed in most T lymphocytes, memory-type B cells and NK cells and appears to be critical for effector and memory differentiation [55]. It enhances the accumulation of newly activated T cells [56] and amplifies the proliferation of purified T lymphocytes to suboptimal stimulation [57]. CD27 expression is down-regulated by mycophenolic acid [58].

Our data do not suggest that CD27 is important in human KTx.

We are aware of the limitations of our study. It is a retrospective study with restricted number of samples. Furthermore, as a result of the limited amount of each Fnab we were unable to analyse the whole set of costimulatory molecules in the same sample. Also, following the restrictions inherent to human studies a sequential sampling of the same KTx could not be performed.

This acknowledged we believe our data are rather consistent and produce further information on the understanding of the allograft response. They highlight the potential important role CD26 may play in deploying anti-allograft response, they suggest ICOS is involved in acute rejection independently of the rejection type, and confirm the importance of $CD16^+$ cells in human renal transplants. Provided our results are reproduced in a larger number, some of these costimulatory molecules could enter the package of diagnostic tools in KTx, especially trough analysis of FNAB by flow cytometry as we have previously reported, while at the same time, identifying steps that could be modulated in order to achieve a betterment of current immunosuppressive protocols.

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Authorship

Paula Xavier co-designed the research study, performed laboratory work, analysed data and co-wrote the paper. José G. G. Oliveira co-designed the research study, performed aspiration biopsies and laboratory work, collected data and co-wrote the paper.

Conflicts of Interest

The authors declare no conflicts of interest.

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Abbreviations

Fine-needle aspiration biopsies: Fnab

Kidney transplants: KTx