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Brief Communication

Trends in Immune Cell Function Assay and Donor-Specific HLA Antibodies in Kidney Transplantation: A 3-Year Prospective Study

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The immune cell function assay (ICFA) and *de novo* anti-donor-specific HLA antibodies (DSA) have been proposed as assays for immune monitoring in renal transplantation, but longitudinal studies examining the modification of both parameters over time and their relation with clinical events are lacking. We prospectively measured longitudinal changes in ICFA and DSA levels in 55 kidney transplant recipients over 3-year follow-up (534 visits) and analyzed their relation with the risk of developing acute rejections or infections. Seven patients (12.7%) developed biopsy-proven acute rejection, and 20 (36.4%) developed viral infections. At 3 years posttransplant, 28% of the patients had developed *de novo* DSA. ICFA levels peaked at 1–2 months posttransplant ($p = 0.005$) and leveled off thereafter. They were not associated with the risk of acute rejections, viral infections or development of *de novo* DSA. Instead, the incidence of *de novo* DSA was higher in patients who previously had viral infections (adjusted-odds ratio of *de novo* DSA associated with prior infections: 6.03 [95% CI, 1.64–22.06; $p = 0.007$]). Our prospective, longitudinal study does not support using ICFA to quantify the immune risk in kidney transplantation. Further studies are needed to confirm the relationship between viral infections and the subsequent development of *de novo* DSA.

Keywords: Anti-HLA antibody, ImmuKnow, kidney transplant

Abbreviations: BAF, background adjustment factor; CMV, cytomegalovirus; DSA, donor-specific HLA antibodies; eGFR, estimated glomerular filtration rate; FDA, Food and Drug Administration; HLA, human leukocyte antigen; ICFA, immune cell function assay; MFI, median fluorescence intensity; MMF, mycophenolate mofetil; PHA, phyto-hemagglutinin-L; PRA, panel reactive antibody; ROC, receiver operating characteristic; SAB, single antigen bead; Tac, tacrolimus

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Introduction

Assays to determine immune status after kidney transplant are a vivid area of research, since they might help in tailoring immunosuppressive therapy to the single patient need, avoiding the risks associated with over- or under-immunosuppression. In 2002, the United States Food and Drug Administration approved the Cylex® ImmuKnow™ (Cylex, Inc., Columbia, MD), an immune cell function assay (ICFA) for assessing cell-mediated immunity in immunocompromised individuals (1). The test measures the increase in ATP production by $CD4^+$ T cells after stimulation with mitogen phyto-hemagglutinin-L (PHA) *in vitro* (2) and could potentially provide information on T cell alloreactivity status that cannot be obtained from the mere assessment of drug blood levels. Some cross-sectional or follow-up retrospective studies have shown that single-point ICFA can identify patients at risk of developing infection (with low ATP levels), or acute rejection (with high ATP levels) in organ transplant patients (3). A recent meta-analysis, however, argued against its predictive power, especially with regard to acute rejection (4).

Measurement of *de novo* donor-specific HLA antibodies (DSA) has been recently proposed as another important tool to monitor the humoral counterpart of the alloimmune status. Development of alloantibodies has been associated with worse graft outcomes and is thought to represent a sign of poor immunosuppression (5–7).

However, longitudinal studies (i.e. studies with repeated measurements over time) on jointly measured *de novo* DSA and ICFA levels are still lacking. The present study sought to

examine the longitudinal change of $CD4^+$ T cell function (measured by ICFA) and the occurrence of *de novo* DSA after kidney transplantation and their relationship with clinical events.

Methods

Study population

This prospective study included all consecutive patients who underwent a single kidney or simultaneous kidney–pancreas transplant at the Parma Renal Transplant Centre, Parma, Italy, during Monday to Thursday (for logistical reasons), from May 1, 2008 to September 1, 2010. Before inclusion, eligible patients provided written informed consent to study participation according to the Declaration of Helsinki. A negative NIH CDC T cell crossmatch was required prior to transplantation.

Patient management

After kidney transplantation, all patients were managed according to the standard protocol in use at our center. Immunosuppressive therapy consisted of induction with thymoglobulin (1.25–1.50 mg/kg for 4–5 doses) or basiliximab (20 mg on day 0 and 4). Maintenance immunosuppressive treatment was based on steroids, tacrolimus (Tac; target trough levels measured by antibody-conjugated magnetic immunoassay: 6–12 μg/L, then 5–10 μg/L from day 30 posttransplant) and mycophenolate mofetil (MMF; 1 g twice daily [b.i.d.], tapered over 1 month to 500 mg b.i.d.). A minority of patients were treated with cyclosporine microemulsion (Neoral), in association with MMF 1 g b.i.d. or with everolimus (target trough levels 8–12 μg/L); target cyclosporine blood levels were 100–150 μg/L (trough) and 200–400 μg/L (2 h postdose), respectively.

All patients were given standard anti-microbial prophylaxis, including trimethoprim-sulfamethoxazole against *Pneumocystis jirovecii* for 6–12 months posttransplant.

Cytomegalovirus (CMV) reactivations were monitored by measuring *pp65* antigen in leukocytes or quantitative nucleic acid testing in plasma. All patients were also routinely screened for BKV with quantitative urine and plasma nucleic acid testing at 1, 2, 3 and 12 months posttransplant. The presence of BKV viremia was followed by 50% dose reduction or complete suspension of MMF. If viremia failed to start clearing within 3–4 weeks despite MMF withdrawal, the calcineurin inhibitor was also tapered, typically targeting trough levels of 4–6 μg/L and 100–150 μg/L for tacrolimus and cyclosporine, respectively. The decision to reduce immunosuppressive therapy after other infections such as CMV or other herpes virus infections (e.g. HVZ infection) was at the discretion of the transplant physician who was in charge of the patient care.

ICFA and Luminex single antigen bead monitoring

ICFA and Luminex single antigen bead (SAB) were measured immediately before transplant surgery, at 1, 2, 3, 6, 9 and 12 months, and every 6 months thereafter, up to month 36 after transplantation. These measurements were blinded to the attending physician who was in charge of the patient care.

Immune cell function assay: Immune function was determined using the Cylex® ImmuKnow™ Cell Function Assay Kit according to the manufacturer's instructions.

Briefly, 100 microL of whole blood was incubated with and without PHA for 15–18 h at 37°C. $CD4^+$ cells were then recovered by positive selection with anti-CD4 magnetic beads (Dynabeads; Dynal, Oslo, Norway). Bead-isolated cells were washed and lysed to release intracellular ATP, which was measured using the luciferin/luciferase system in a luminometer (Turner BioSystems, Sunnyvale, CA). The concentration of intracellular ATP (ng/mL) in each sample was calculated from a calibration curve prepared using ATP calibrators (0, 1, 10, 100 and 1000 ng/mL). Replicate samples with a calculated percentage coefficient of variation greater than 20% were included in the calculation if a single value was within 3 standard deviations (SDs) of the mean value of all wells.

Panel reactive antibody screening and identification test: Panel reactive antibody (PRA) screening and SAB assay were performed using Luminex assay kits (LIFECODES; Gen-Probe, Stamford, CT) according to the manufacturer's guidelines. Only samples that tested positive for PRA screening were further tested for the single antigen identification of Class I and/or Class II antibodies. Test samples were analyzed on the Luminex IS100 instrument (Luminex, Austin, TX), and data were analyzed using Quick Type software (Gen-Probe, Stamford, CT).

In the SAB assay, median fluorescence intensity (MFI) for the individual bead was divided by the MFI of three negative control beads and a fourth negative control calculation. From these quotients, the background adjustment factor was subtracted. A positive value for two or more of the four calculations indicated a positive bead reaction, and a negative value for three or more of the four calculations indicated a negative bead reaction. The baseline MFI value that was used to identify antibody specificities was 2000.

Data analyses

Nonrepeated continuous and categorical unpaired data were compared by unpaired t-test, Mann–Whitney test and Fisher's exact test, as appropriate unless otherwise stated. To account for the presence of unbalanced data, we analyzed ICFA and Tac levels with repeated-measures linear mixed-models using restricted maximum likelihood (8). We used random coefficient regression models to estimate changes over time in the presence of unbalanced data (8). The relations between the incidence of *de novo* DSA and ICFA, Tac levels and infections were examined by multiple logistic regression using generalized estimating equations with robust standard errors to account for repeated measurements in each patient (8,9). We used the Stata programs *roctab* and *diagt*, with the default standard errors, to compute 95% confidence intervals for the area under the receiver operating characteristic (ROC) curve (10), and for sensitivity, specificity, likelihood ratios and diagnostic odds ratios (11). A two-sided α-level of 0.05 was used for statistical significance. All analyses were performed using Stata Statistical Software package, Release 12.0. (StataCorp, College Station, TX).

Results

Baseline characteristics

The patient population consisted of 55 Caucasian adult recipients of kidney ($n = 53$) or simultaneous kidney–pancreas ($n = 2$) transplants, mainly from deceased donors (Table 1). Twenty-five patients received induction therapy with thymoglobulin and 29 with basiliximab. Most patients received maintenance immunosuppression with steroids, Tac and MMF (Table 1). Both recipients of ABO-incompatible living donor kidney transplants were treated with rituximab (375 mg/m^2, single dose) 28 days before surgery.

Follow-up

Death and graft loss: Two patients died because of pancreas cancer and spontaneous perforation of the colon

Table 1: Baseline patients' characteristics

Characteristics of patients (n = 55)	
Number of visits	534
Visits per patient, median (range)	10 (6–11)
Recipient's age	48.9 ± 12.4
Male gender	33 (60.0)
Pretransplant ICFA (ng/mL)	264.5 ± 138.9
Type of transplant	
Deceased kidney donor	38 (69.1)
Living kidney donor	15 (27.3)
ABOi-living donor kidney	2/15 (13.3)
Simultaneous pancreas–kidney	2 (3.6)
HLA mismatch level	
Level 1	0 (0.0)
Level 2	17 (30.9)
Level 3	28 (50.9)
Level 4	10 (18.2)
Highly sensitized recipient	1 (1.8)
Pretransplant DSA	2 (3.6)
Induction treatment	
Thymoglobulin	25 (45.5)
Basiliximab	29 (52.7)[a]
Rituximab	2 (3.6)[b]
Maintenance treatment	
Tac + MMF + GC	48 (87.3)
CyA + MMF + GC	4 (7.3)
Everolimus + CyA + GC	3 (5.5)

ABOi, ABO-incompatible living donor kidney transplant; CyA, cyclosporine microemulsion (Neoral); DSA, donor-specific HLA antibodies; GC, glucorticoids; ICFA, immune cell function assay; MMF, mycophenolate mofetil. Highly sensitized, CDC panel reactive antibody >80% in at least two occasions.
Data are reported as number (percentage) or mean ± standard deviation, unless otherwise specified.
HLA mismatch level was defined as follows: HLA-A, HLA-B and HLA-DR loci: level 1 was a 000 HLA-A, HLA-B and HLA-DR mismatch; level 2 was a 0 HLA-DR plus 0/1 HLA-B mismatch; level 3 was a 0 HLA-DR plus 2 HLA-B mismatch or a 1 HLA-DR plus 0/1 HLA-B mismatch; and level 4 was a 2 HLA-DR or a 1 HLA-DR plus 2 HLA-B mismatch.
[a]One recipient of ABOi-living donor transplant received neither thymoglubulin nor basiliximab induction.
[b]Rituximab was used in the two recipients of an ABOi-living donor kidney transplant.

after 1.9 and 2.2 years, respectively. One patient lost the graft at 1 year following immunosuppression tapering after an episode of concomitant BKV and CMV infection (acute T cell–mediated Type IIB rejection).

Tac levels and graft function: Tac trough levels were within the targeted range both during the first 3 months posttransplant, and during the period 6–36 months (7.68 ± 0.21 vs. 7.83 ± 0.21 μg/L).

Acute and chronic rejection: Over the follow-up period, seven patients (12.7%) developed acute rejection (Table 2). Three additional patients developed chronic antibody-mediated rejection, two of them with evidence of transplant glomerulopathy. One early and reversible T cell–mediated rejection (Type IA) occurred in the ABO-incompatible transplant, whereas no rejection occurred in the highly sensitized patient.

Table 2: Acute and chronic rejections and infections

Clinical outcome	
Acute rejection (all biopsy-proven)	
All	7 (12.7)
Banff T cell–mediated Type IA/IB	3 (5.5)
Banff T cell–mediated Type IIA/IIB	2 (3.6)
Antibody-mediated or mixed	2 (3.6)
Chronic rejection (all biopsy-proven)	
All	3 (5.5)
With transplant glomerulopathy	2 (3.6)
C4d positive	1 (1.8)
Infectious complications	
BKV plasma replication	5 (9.1)
BKV-associated nephropathy	1 (1.8)
CMV infection	11 (20.0)
Other HSV infection (all HVZ)	5 (9.1)
Major respiratory tract infections	9 (16.4)
Urinary tract infections	20 (36.4)
Other infections	14 (25.5)

BKV, polyoma BK virus plasma replication; CMV, cytomegalovirus infection; HSV, herpes virus; HVZ, herpes varicella-zoster infection. Major respiratory tract infection, infection requiring hospital admission and/or intravenous antibiotics.
Data are reported as number (percentage).

Infections: Five patients (9.1%) had BKV plasma replication and one of them had BKV-associated nephropathy. CMV infection occurred in 11 patients (20.0%), 2 of whom had concomitant BKV plasma replication. Five patients had other HSV infections that were all Herpes-Zoster infections (Table 2). Overall, 20 of the patients (36.4%) had CMV, BKV or Herpes-Zoster infections.

ICFA levels: ICFA levels peaked at months 1–2 posttransplant and eventually leveled off from months 6 to 36 (average up to month 3: 303.2 ± [standard error] 15.1; average months 6–36: 244.6 ± 13.5 ng/mL; $p = 0.005$ for the difference between the two time periods). The difference between months 0–3 and months 6–36 did not vary according to induction therapy (Figure 1; $p = 0.98$ for the interaction test between the time-contrast and the type of induction).

De novo *DSA:* At the 12-month visit, 10.0% of the patients (5/50; 95% CI: 4.3–21.4) had developed *de novo* DSA (Class I: 4%; DSA Class II: 8%), a fraction that increased up to 28.6% (10/35; 95%CI: 16.3–45.1; Class I: 11.4%; DSA Class II: 25.7%) at the 36-month visit (Figure 2). Over the course of follow-up, patients with *de novo* DSA had numerically lower ICFA levels compared to those who never developed *de novo* DSA (−40.6 ng/mL; $p = 0.16$; Figure 3).

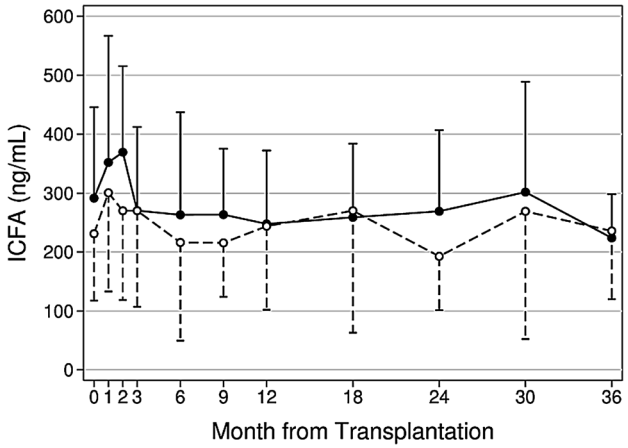

Figure 1: Mean ICFA (connected dots) and standard deviation (vertical bars) in patients receiving basiliximab induction (solid circles and solid line) and in patients receiving thymoglobulin induction (hollow circles and dotted line). The difference between the average of month 0–3 and month 6–36 ICFA was similar between basiliximab and thymoglobulin ($p = 0.98$). ICFA, immune cell function assay.

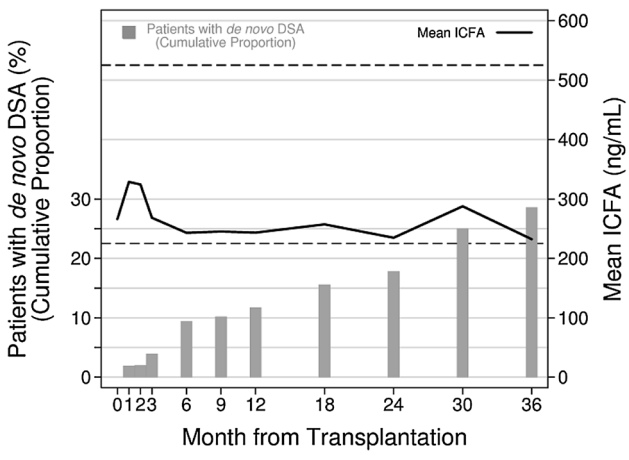

Figure 2: Average ICFA levels (solid black line), cutoffs for defining low and strong immune response by ICFA (lower and upper horizontal dash lines, respectively), and cumulative proportion of patients who had developed *de novo* DSA over time (gray bars). Despite the average study population ICFA levels remaining close to the ICFA lower cutoff (indicating ''low immune cell response'') over the entire course of follow-up, there was a relentless increase in the proportion of patients who had developed *de novo* DSA. The scale of the left y-axis refers to the proportion of *de novo* DSA, the scale of the right y-axis refers to the ICFA levels. DSA, donor-specific HLA antibodies; ICFA, immune cell function assay.

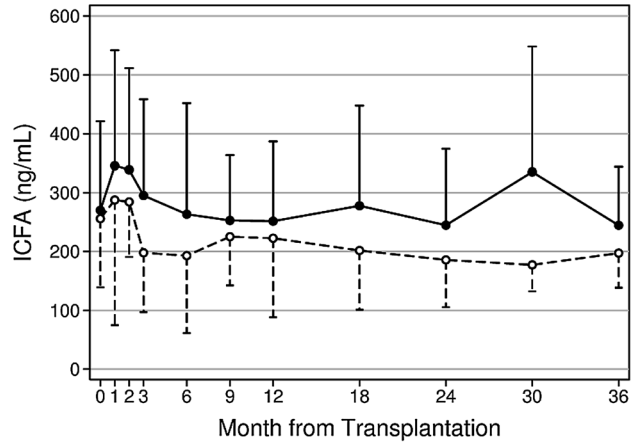

Figure 3: Mean ICFA (connected dots) and standard deviation (vertical bars) in patients who never develop *de novo* DSA (solid circles and solid line) and in patients who developed *de novo* DSA (hollow circles and dotted line). ICFA levels over follow-up were numerically lower in patients who developed *de novo* DSA, but the difference between the groups was not statistically significant ($p = 0.16$). DSA, donor-specific HLA antibodies; ICFA, immune cell function assay.

Relations between ICFA levels and clinical parameters

Tac levels: There was no correlation between ICFA levels and Tac blood concentrations during follow-up ($p = 0.56$), as well as between intra-subject variability of ICFA and Tac levels (Spearman rank correlation coefficient between intra-subject standard deviations: +0.11; $p = 0.46$).

Acute rejection episodes: The crude findings are reported in Table 3. After adjusting for time from transplant, type of transplant, thymoglobulin induction, type of maintenance immunosuppressive treatment and HLA mismatch level, there was no significant difference in ICFA between patients with acute rejection and those without acute rejection (+42.8 ng/mL [95% CI, −39.2 to +124.7; $p = 0.31$]). The measurements in patients with rejection episodes included in the analysis were only those taken *before* the rejection episode. When we additionally adjusted for Tac blood levels (in the subset of 48 patients taking Tac), the analysis yielded similar results (+101 ng/mL [95%CI, −32.0 to +234.9; $p = 0.14$]). Similarly, the time trend in ICFA levels prior to rejection did not differ compared to the patients with and without acute rejection (data not shown). The analysis also showed that adjusted Tac blood levels prior to rejection did not differ between patients who had or not acute rejection (+2.4 ng/mL; $p = 0.77$).

Infection episodes: The crude findings are reported in Table 3. The adjusted analyses showed that ICFA levels did not differ between patients with or without viral infections (for patients with infections, we considered samples taken *before* the infection episodes).

Predictive capacity for acute rejection and infection: Consistent with the findings reported above, the point

Table 3: Associations between major clinical events and clinical characteristics at baseline or over the follow-up

	Acute rejection		Acute or chronic rejection		*De novo* DSA[a]		BKV, CMV or HSV infections		Viral or major resp. tract infections	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
n	7	48	10	45	15	38	20	35	26	29
ICFA, ng/mL	327 ± 88	253 ± 78	282 ± 135	254 ± 80	224 ± 56	276 ± 100	290 ± 134	259 ± 98	298 ± 129	262 ± 99
Tac use	7 (100)	41 (85)	10 (100)	38 (84)	13 (87)	34 (89)	20 (100)	28 (80)	26 (100)	22 (76)[b]
Tac level, μg/L[c]	7.4 ± 2.5	7.0 ± 1.0	7.2 ± 0.9	6.9 ± 1.0	7.2 ± 1.0	6.9 ± 1.0	7.4 ± 1.8	7.1 ± 0.9	8.0 ± 2.2	7.0 ± 0.8[b]
MMF use	6 (86)	45 (84)	9 (90)	42 (93)	12 (80)	38 (100)[b]	20 (100)	31 (89)	26 (100)	25 (86)
Thymoglobulin	2 (29)	23 (48)	3 (30)	22 (49)	5 (33)	19 (50)	9 (45)	16 (46)	13 (45)	12 (46)
HLA mm level	3.4 ± 0.5	2.8 ± 0.7[b]	3.2 ± 0.6	2.8 ± 0.7	3.1 ± 0.5	3.8 ± 0.7	3.0 ± 0.7	2.8 ± 0.7	3.0 ± 0.7	2.8 ± 0.7
Deceased donor transplant	2 (29)	38 (79)[b]	4 (40)	36 (80)[b]	9 (60)	30 (79)	25 (71)	15 (75)	20 (77)	20 (70)
ABOi-living kidney donor	1 (14)	1 (2)	1 (10)	1 (2)	2 (13)	0 (0)	1 (5)	1 (2)	1 (4)	1 (3)
SPK	1 (14)	1 (2)	1 (10)	1 (2)	0 (0)	2 (5)	1 (5)	1 (3)	2 (8)	0 (0)

ABOi, ABO-incompatible living donor kidney transplant; CyA, cyclosporine microemulsion (Neoral); DSA, donor-specific HLA antibodies; GC, glucorticoids; ICFA, immune cell function assay; MMF, mycophenolate mofetil. Highly sensitized, CDC panel reactive antibody >80% in at least two occasions.

Data are reported as number (percentage) or mean ± standard deviation.

ICFA mean of patients' average over the course of follow-up (therefore only one value per subject was used for the computations reported in the Table). For acute complications such as Acute rejection, BKV, CMV or HSV infections, and Viral or major resp. tract infections, the average is computed using only the values taken prior to the event.

Tac level, tacrolimus blood level mean of patients' average of over the course of follow-up (therefore only one value per subject was used for the computations reported in the Table). For acute complications such as Acute rejection, BKV, CMV or HSV infections, and Viral or major resp. tract infections, the average is computed using only the values taken prior to the event.

HLA mm level, HLA mismatch level that was defined as follows: HLA-A, HLA-B and HLA-DR loci: level 1 was a 000 HLA-A, HLA-B and HLA-DR mismatch; level 2 was a 0 HLA-DR plus 0/1 HLA-B mismatch; level 3 was a 0 HLA-DR plus 2 HLA-B mismatch or a 1 HLA-DR plus 0/1 HLA-B mismatch; and level 4 was a 2 HLA-DR or a 1 HLA-DR plus 2 HLA-B mismatch.

[a]The two patients with pretransplant DSA were not used in the computations.

[b]$p < 0.05$ (by Fisher's exact test or Mann–Whitney test).

[c]Tac level was computed only in the subset of 48 taking tacrolimus.

Table 4: Summary statistics describing ICFA predictive capacity for acute rejection and for acute infections during the study period

	Acute rejection	BKV, CMV or HSV infections	Viral or major resp. tract infections
Any ICFA value beyond the diagnostic threshold during follow-up			
Sensitivity, %	28.6 (3.7–71.0)	63.2 (38.4–83.7)	69.2 (48.2–85.7)
Specificity, %	70.8 (55.9–83.0)	5.7 (0.7–19.2)	6.9 (0.8.22.8)
Positive likelihood ratio	0.98 (0.28–3.42)	0.67 (0.47–0.95)	0.74 (0.56–0.98)
Negative likelihood ratio	1.01 (0.61–1.67)	6.45 (1.48–28.0)	4.46 (1.04–19.1)
Diagnostic odds ratio	0.97 (0.0–4.99)	0.10 (0.00–0.52)	0.17 (0.0–0.79)
Area under the ROC curve[a]	0.44 (0.18–0.71)	0.34 (0.17–0.63)	0.37 (0.22–0.53)
ICFA cumulative average beyond the diagnostic threshold at last follow-up			
Sensitivity, %	0.0 (0.0–41.0)	36.0 (18.0–57.5)	36.8 (16.3–61.6)
Specificity, %	97.9 (88.9–99.9)	62.1 (42.3–79.3)	60.0 (42.1–76.1)
Positive likelihood ratio	NA	0.95 (0.47–1.91)	0.92 (0.45–76.1)
Negative likelihood ratio	1.02 (0.98–1.06)	1.03 (0.68–1.55)	1.05 (0.68–1.63)
Diagnostic odds ratio	NA	0.92 (0.31–2.74)	0.87 (0.28–2.71)
Area under the ROC curve	0.69 (0.46–0.93)	0.45 (0.28–0.63)	0.45 (0.28–0.61)

BKV, polyoma BK virus plasma replication; CMV, cytomegalovirus infection; HSV, herpes virus; NA, not available (i.e. the ratio cannot be calculated); ROC, receiver operating characteristic. Major respiratory tract infection, infection requiring hospital admission and/or intravenous antibiotics.

Summary statistics (and 95% confidence intervals) describing ICFA predictive capacity for acute rejection and infection. The follow-up used for the analyses consists in all the visits up to the last one preceding the acute event, or up to the end of the study period. The ICFA diagnostic thresholds are $\geq$525 and $\leq$225 ng/mL for acute rejection and for acute infection, respectively. The summary statistics of the upper part of the table were computed after having classified patients according to whether or not they had an ICFA beyond the threshold at any time during follow-up; the summary statistics of the lower part of the table was computed after having classified patients based on the ICFA cumulative average at the last follow-up visit. The cumulative average is the ICFA mean of all visits up to and including the last one.

The 95% confidence interval including the null value indicates that ICFA has no predictive capacity. The null value is 0.50 for area under the ROC curve, and is 1.0 for Likelihood Ratios and Diagnostic Odds Ratios. Because ICFA values were often smaller in patients with acute rejection compared to patients without acute rejection and were often larger in patients with infection compared to patients without infection, virtually all the point estimates fall on the wrong side about the null value (i.e. the area under the ROC curve < 0.5, the positive likelihood ratio > 1.0, the negative likelihood ratio 1.0 and the diagnostic odds ratio < 1.0).

[a]This ROC curve was computed using the highest and the lowest ICFA during follow-up, for acute rejection and acute infections, respectively.

estimates and the 95% confidence intervals for the likelihood ratios, the diagnostic odds ratios (evaluated at the standard diagnostic cutoff limits of 225 ng/mL and 525 ng/L), and of the area under the ROC curve did not provide evidence that ICFA has a predictive capacity for future clinical events (Table 4, see legend for a detailed explanation).

Relations between de novo *DSA and clinical parameters*

Tac levels: During the whole follow-up period, Tac blood levels were virtually identical in patients who developed and in those who did not develop *de novo* DSA ($p = 0.37$).

Acute rejection episodes: Out of the 53 patients with no pretransplant DSA, *de novo* DSA occurred in 50.0% (3/6) of those who had acute rejection (MFI 14 613, 11 984, and 23 201, respectively) and 25.5% (12/47) of those who did not develop acute rejection (median MFI 3143 [range 2564–8422]) (differences between-groups: for the incidence of *de novo* DSA: $p = 0.33$; for MFI levels: $p = 0.002$, by the exact rank-sum test). Of the two patients with pretransplant DSA, one developed acute antibody-mediated rejection.

Infection episodes: The incidence of *de novo* DSA was 45.0% (9/20) and 18.2% (6/33) in patients who did and did not develop infections, respectively ($p = 0.058$). Figure 4 reports follow-up of the nine patients with infection and DSA.

After adjusting for thymoglobulin induction, type of transplant, HLA mismatch number, time from transplantation (months 0–1, 2–6, 9–36), ICFA levels and Tac levels during follow-up, the odds ratio of *de novo* DSA associated with *previous* occurrence of viral infections was 6.03 (95% CI, 1.64–22.06; $p = 0.007$). Figure 5 reports adjusted probabilities of *de novo* DSA based on the multiple regression estimates. The association between viral infections and subsequent development of *de novo* DSA remained statistically significant after including in the analysis the seven patients not taking Tac, after excluding from the analysis the seven patients who developed acute rejections, and also after excluding the two patients who had pretransplant DSA (data not shown). Results were also similar after including, among the episodes of infections, episodes of major respiratory tract infection (i.e. requiring admission and/or intravenous antibiotics, data not shown).

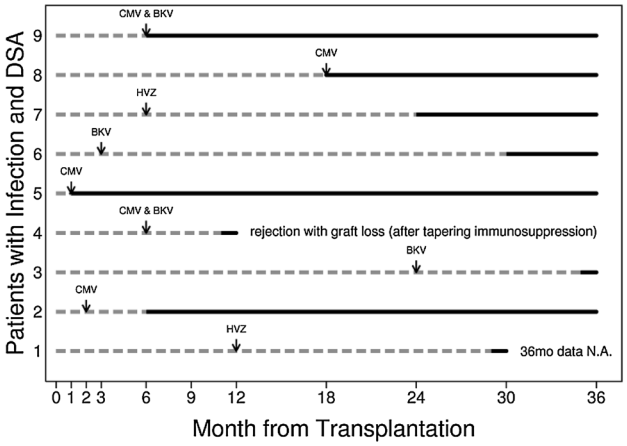

Figure 4: Time of onset of viral infection and of development of *de novo* DSA in the nine patients who had both infection and *de novo* DSA during follow-up. Each line represents a patient. The dashed lines become solid at the time when a *de novo* DSA was first detected. All patients developed HLA Class II donor-specific antibodies. Patients 3 and 8 also developed HLA Class I donor-specific antibodies. At the time of infection seven of the nine patients changed MMF administration: Patients 4, 5, 6, 8 and 9 withdrew MMF altogether, whereas patients 2 and 3 halved the dose. Overall, average ± standard deviation Tac blood levels were 7.8 ± 2.4 μg/L before infection and 6.6 ± 2.1 μg/L after infection ($p = 0.093$, by Wilcoxon-signed ranks test). Patient 4 lost the graft because of Type II rejection (with subsequent appearance of DSA) after tapering of immunosuppression. Patient 1 did not attend the 36-month visit. BKV, polyoma BK virus plasma replication; CMV, cytomegalovirus infection; DSA, donor-specific HLA antibodies; HVZ, herpes varicella-zoster infection; MMF, mycophenolate mofetil.

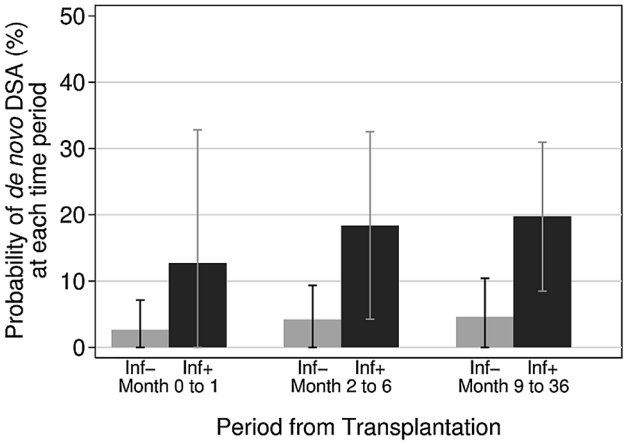

Figure 5: Predicted risk of developing *de novo* DSA at different time periods from transplant (months 0–1, 2–6, and 9–36, respectively) in patients free of viral infections (gray bars) and in those who suffered from viral infections (black bars). The vertical lines associated with each bar denote 95% confidence intervals. The predictions are computed on the basis of the estimates from multiple logistic regression using generalized estimating equation models which were adjusted for ICFA levels, tacrolimus levels, thymoglobulin use, HLA mismatch levels and type of transplant. The probability of *de novo* DSA increased significantly in patients who had previous viral infection ($p = 0.007$), irrespective of the time elapsed from transplantation. DSA, donor-specific HLA antibodies; ICFA, immune cell function assay; Inf, viral infection.

Discussion

This prospective, longitudinal study showed that ICFA levels had a transient up-regulation during the first 3 months after kidney transplantation with a tendency to become stable below baseline values during the following 3 years. We could find no association between increased ICFA levels and the subsequent acute rejection and development of *de novo* DSA, or between decreased ICFA levels and subsequent infections. On the other hand, development of *de novo* DSA was associated with previous occurrence of viral infections.

Initial reports showed that ICFA values decrease in the setting of active, clinically apparent infection, and increase with clinical improvement in disease (3,12,13). However, these studies measured the ICFA values at the time of disease rather than before the onset of disease. Other authors have attempted to assess ICFA as a predictive test and have reported that low ICFA values are associated with an increased incidence of infections in stable transplant recipients on maintenance immunosuppression (3,14–16). Yet, these studies were small with relatively few infection episodes and short follow-up periods. A subsequent large, retrospective analysis of 1330 ICFA values in 583 renal transplant recipients at a single center failed to detect an association between preceding ICFA levels and future development of opportunistic infections or acute rejections in the following 3 months (17). A recent meta-analysis reached a similar conclusion challenging the power of ICFA to predict the occurrence of opportunistic infections or acute rejections in solid organ transplantation, and also provided statistical evidence of publication bias related to the underreporting of studies showing low ICFA predictive capacity (4).

Our current study supports and expands these findings in the setting of a 3-year prospective longitudinal study. By taking advantage of the repeated measurement design, we examined whether ICFA levels, measured at fixed time points during follow-up, could herald the occurrence of clinical complication such as rejection, infections or the occurrence of *de novo* DSA. However, after having performed in-depth analyses of the data, we could not find any significant relation. We also failed to detect any relationship between ICFA and Tac blood levels.

The early increase in ICFA levels observed in our cohort resembles that previously described by others (18) in patients receiving thymoglobulin induction, an effect that was attributed to the high metabolic activity of

postdepletion $CD4^+$ T cells. Though understanding the mechanistic explanation for this phenomenon was beyond the purpose of the present study, our findings challenge this hypothesis, since the same increase in ICFA levels was found in patients induced with nondepleting anti-CD25 therapy, and highlight a seemingly consistent T cell metabolic change in the early posttransplant period that could be important in the establishment of alloimmune response. On the other hand, our findings are also important in interpreting the results of ICFA itself when they are used for predicting future clinical events.

Despite average ICFA levels in the study population that remained close to the ICFA lower threshold, indicating potential overimmunosuppression (Figure 2), there was a relentless increase in the incidence of *de novo* DSA. Importantly, incidence of DSA was associated with previous viral infections, supporting the intriguing hypothesis that infectious episodes trigger B cell activation (19,20). However, larger studies are needed to confirm our findings.

We acknowledge that our study has some limitations. The relatively limited sample size and the few clinical events limited the power of the study. However, patients were strictly followed up in a repeated measures study, and the number of dropouts was minimal. Strength of our findings is further supported by the fact that the investigators who performed the assays and those who were in charge of patients were blinded to each other's data.

In summary, ICFA does not appear to be a valuable assay to predict future occurrence of infections or acute rejections in kidney transplantation, whereas development of *de novo* DSA had an association with viral infections, which must be confirmed by further studies.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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