TNF-α Production by Monocytes Stimulated With Epstein-Barr Virus–Peptides as a Marker of Immunosuppression-Related Adverse Events in Kidney Transplant Recipients

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Introduction: Infections and cancers now outnumber rejection as a cause of morbidity in transplant recipients, likely as a result of over-immunosuppression. Currently, there is no clinical tool to detect over-immunosuppression. We recently reported that tumor necrosis factor alpha (TNF-α) production by $CD14^+CD16^+$ intermediate monocytes, following *ex vivo* stimulation by Epstein-Barr virus–peptides, could identify over-immunosuppressed patients.

Methods: We conducted a pilot study the assay using 142 peripheral blood mononuclear samples from a cohort of 71 kidney transplant recipients. Patients were classified as cases or controls according to the occurrence of opportunistic infection, recurring bacterial infections or *de novo* neoplasia in the 12 months following blood collection. We used both the classifier rule and a threshold of $<73\%$ of $CD14^+CD16^+TNF\alpha^+$ cells developed in a previous training set.

Results: Cases were detected with 83% sensitivity and 68% specificity. The negative predictive value of the assay was 89%. The hazard ratio for the occurrence of the endpoint was 6.8 (95% confidence interval 2.0–23.9; $P = 0.003$) in patients with a positive test. Multivariable linear regression analysis revealed that the association was independent of baseline clinical characteristics, renal function, and immunosuppressive regimen.

Conclusion: These data validate this cell-based assay as a promising tool for personalizing immunotherapy. Studies are under way for a 2-step assay with improved specificity.

The observed increase in kidney graft survival over the past decades is largely due to improved immunosuppression.[1–3] As a result, the rate of adverse effects due to immunosuppressants exceeds the rate of rejection.[4,5] A *post hoc* analysis of the FAVORIT trial demonstrates that infection and malignancy now outweigh cardiovascular mortality in kidney recipients.[6] Clinically, finding the right balance between under- and over-immunosuppression (OIS) is difficult. Current guidelines on immunosuppressant dosing are based on patients' blood drug levels, anthropomorphic data, and medical history, which lack precision. Importantly, they do not account for patients' heterogeneity in the *in vivo* response to immunosuppressants.

The net state of immunosuppression refers to the capacity of the immune system to build a competent response to infection or cancer.[7,8] In transplant recipients, the immunosuppressive load is obviously a major factor influencing this state.[8] Adequate monitoring of the net state of immunosuppression is a yet unachieved goal. Although biomarkers for acute rejection are currently on their way to be translated to the clinic, there remains no reliable clinical tool to distinguish immune-competent patients from those who are in a state of OIS. As stated recently by Thaunat,[9] complications are indeed the current marker of OIS.

Recently, our laboratory reported that impaired secretion of tumor necrosis factor alpha (TNF-α) by

intermediate monocytes (CD14$^+$CD16$^+$) stimulated with Epstein-Barr (EBV) peptides identified OIS in *de novo* kidney recipients.[10,11] This observation is biologically plausible because monocytes play a key role in innate immune defense, while also contributing to activation of T cells by antigen presentation.[12,13] T-cell depletion studies have revealed that the monocyte response is dependent on T-cell recognition of EBV peptides, confirming the capacity of this test to measure amplified T-cell responses.[10] The aim of the present study was to further validate this cell-based assay in an independent, external cohort of kidney recipients.

METHODS

Study Design and Population

This is a single-center, observational cohort study based on 146 consecutive peripheral blood mononuclear cell (PBMC) samples collected between January 2015 and August 2017 from 73 adult kidney graft recipients (Figure 1). Any kidney transplant recipient aged at least 18 years was offered to participate in a prospective biobank at the time of a protocol or indication biopsy. Participation implied collection of clinical data and biological samples on the day of the biopsy (time 0) and 3 months thereafter. Patients could be re-enrolled in the biobank if at least 12 months had elapsed from the first enrollment. This was the case of 2 patients. Patients were excluded when PBMC mortality on thawing was $\geq 30\%$ in 1 or both samples ($n = 2$). The study was approved by the local ethics committee (protocol A12-06-1001, project 2017-3048) and is consistent with the Principles of the Declaration of Istanbul. Written informed consent was obtained from all participants at the time of enrollment.

Sample Collection and Monitoring

Patients were asked to undergo a blood test at each of the 2 study visits. Detailed anthropomorphic and clinical data, including demographics, kidney function, medical diagnoses (infections, rejection, cardiovascular events, and cancer), complete blood counts, and mediation (doses and blood levels) were recorded prospectively at each visit. Modification of immunosuppressant doses and new medical diagnoses, in particular, infections, neoplasia, and rejections, were retrospectively gathered from clinical files for each patient at the end of the 12-month follow-up period. All patients were followed at the transplant center for the duration of the study, and the occurrence of events was recorded prospectively on an annual basis. No patient was lost to follow-up and no patient withdrew consent. Routine serial monitoring for viremia (BK virus, John Cunningham virus, EBV, and cytomegalovirus) was described previously.[10]

Primary Outcome and Patients' Status

The primary outcome was the occurrence of an OIS event. The definition, consistent with previous reports,[14] was a composite of the following: an opportunistic infection, recurring infections (at least 3 episodes) in the absence of a predisposing factor, or *de novo* cancer. Adjudication of the primary outcome was made by a committee blinded to the test result.

Immunosuppressive Protocol, Treatment of Viremia, and Graft Rejection

Regarding induction, 41 patients received basiliximab, 8 received anti-thymocyte globulin, and 30 did not receive induction treatment (Table 1). The standard maintenance immunosuppression regimen was made of triple therapy. Six patients were on azathioprine instead of mycophenolate, 3 were on cyclosporine, 3 were on leflunomide instead of mycophenolate, and 1 was on sirolimus. The standard immunosuppressive protocol and treatment for viremia were described in a previous report.[10]

Cell Assay

Blood samples were treated as described previously.[10] Briefly, PBMCs were thawed and cultured for 3 hours in serum-free medium (X-VIVO-15; Lonza, Walkersville, MD) containing interleukin-2 (25 U/ml, Peprotech, Rocky Hill, NJ). Adherent cells were detached by pipetting in cold phosphate-buffered saline (4 °C). They were then resuspended in serum-free medium (X-VIVO-15) for overnight culture at 37 °C under 5% CO_2 in round-bottom suspension 96-well plates (Sarstedt, Nümbrecht, Germany) at 4×10^6 cells/ml under the following conditions: resting conditions, 1 μg/ml lipopolysaccharide (Sigma-Aldrich, St-Louis, MO) or EBV-derived peptides (as recommended by the manufacturer; EBV Peptivator consensus; Miltenyi Biotec Inc., Auburn, CA). Following overnight
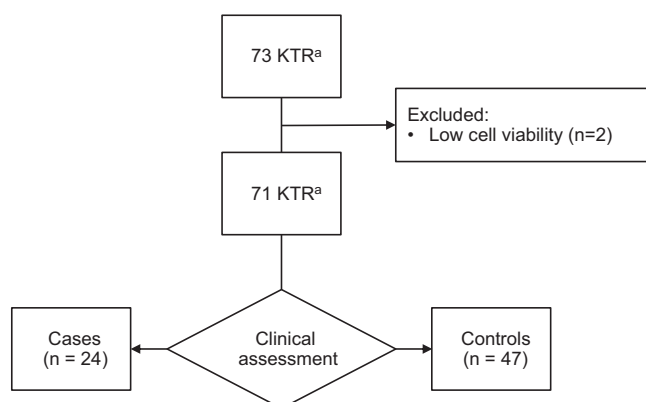
Figure 1. Study flowchart. KTR, kidney transplant recipients. [a] Two patients were enrolled twice, in each case with at least 12 months between enrollments.

Flowchart:
73 KTR[a]
→ Excluded: • Low cell viability (n=2)
71 KTR[a]
→ Clinical assessment
→ Cases (n = 24)
→ Controls (n = 47)

Table 1. Clinical characteristics of the population

	Cases (n = 24)	Controls (n = 47)	P value
Age, yr	60 ± 13	54 ± 13	0.07
Male sex	17 (71)	25 (53)	0.20
First transplant	22 (92)	33 (70)	0.07
Deceased donor	22 (92)	35 (74)	0.12
Time posttransplant, mo	8 [5, 23]	29 [7, 117]	< 0.01
eGFR,[a] ml/min per 1.73 m²	45 ± 15	52 ± 18	0.17
Induction	17 (71)	26 (55)	0.30
ATG	3 (13)	3 (6)	0.40
Basiliximab	14 (58)	23 (49)	0.62
Immunosuppression[a]			
Corticosteroids	24 (100)	47 (100)	–
Prednisone dose, mg	10.1 ± 4.7	7.5 ± 2.6	< 0.01
Calcineurin inhibitor[b]	23 (96)	40 (85)	0.25
Tacrolimus level, ng/dl	7.3 ± 1.8	5.7 ± 1.7	< 0.01
Mycophenolate/AZA[c]	20 (83)	39 (83)	1.00
Mycophenolate dose, mg[d]	1100 ± 522	1088 ± 347	0.88

ATG, anti-thymocyte globulin; AZA, azathioprine; eGFR, estimated glomerular filtration rate.
[a]At time of the initial blood collection.
[b]All patients were on tacrolimus except 1 OIS and 2 controls, who were on cyclosporine.
[c]All patients were on mycophenolate except 6 controls.
[d]In mycophenolate mofetil equivalent.
Data are expressed as mean ± SD, n (%), or median [25th, 75th percentiles]. Renal function was calculated with the Chronic Kidney Disease Epidemiology Collaboration formula. Comparisons were performed using the Mann-Whitney test or Fisher exact test.

culture, PBMCs were incubated for 5 hours with a protein transport inhibitor (GolgiStop; BD Biosciences, San Diego, CA). eFluor 450 staining (Thermo Fischer, Waltham, MA) was used to screen viable cells.[15] Low-viability samples were discarded; this was the case for 2 patients (Figure 1). Fc receptor blocking, extracellular staining, intracellular staining, and fixation were carried out as described previously.[10] The following labeling antibodies were used: anti-CD14-PE-Vio770 (Miltenyi), anti-CD16-APC, and anti-TNF-α-FITC (both BioLegend, San Diego, CA). All antibodies were titrated against their respective isotype. PBMCs were analyzed by flow cytometry on a BD LSR Fortessa instrument (BD Biosciences). Flow cytometry data were analyzed with FlowJo vX software (FlowJo LLC, Ashland, OR). The gating strategy is shown in Supplementary Figure S1. For CD14 and CD16 staining, we used 1% of the isotype as a threshold for positivity. For TNF-α staining, we used 80% positivity on the positive control (lipopolysaccharide) to define the threshold.

Diagnostic Threshold of the Test

Consistent with the classifier rule derived from a previous training set,[10] the test was considered positive for OIS when samples at both month 0 and month 3 had fewer than 73% TNF-α–positive intermediate monocytes.

Statistical Analysis

Clinical variables were examined using Mann-Whitney test or Fisher's exact test, as appropriate. The mean difference for the percentage of TNF-α–positive cells between groups was assessed using the Mann-Whitney test. Cox proportional hazards model was used to derive the risk of the endpoint in relation to the assay result. Violations of the proportional hazards assumption were examined by plots of the logarithm of the negative logarithm of the estimated survivor function versus log time. Univariable and multivariable linear regression models were used to study the association between percentage of TNF-α–positive cells and endpoint. These models relied on the mean of TNF-α percentages at months 0 and 3. Statistical analyses were performed using Stata version 11.0 (StataCorp, College Station, TX) or SPSS Statistics version 25 (IBM, Armonk, NY). All tests were 2-tailed, and the statistical significance threshold was set at 0.05.

RESULTS

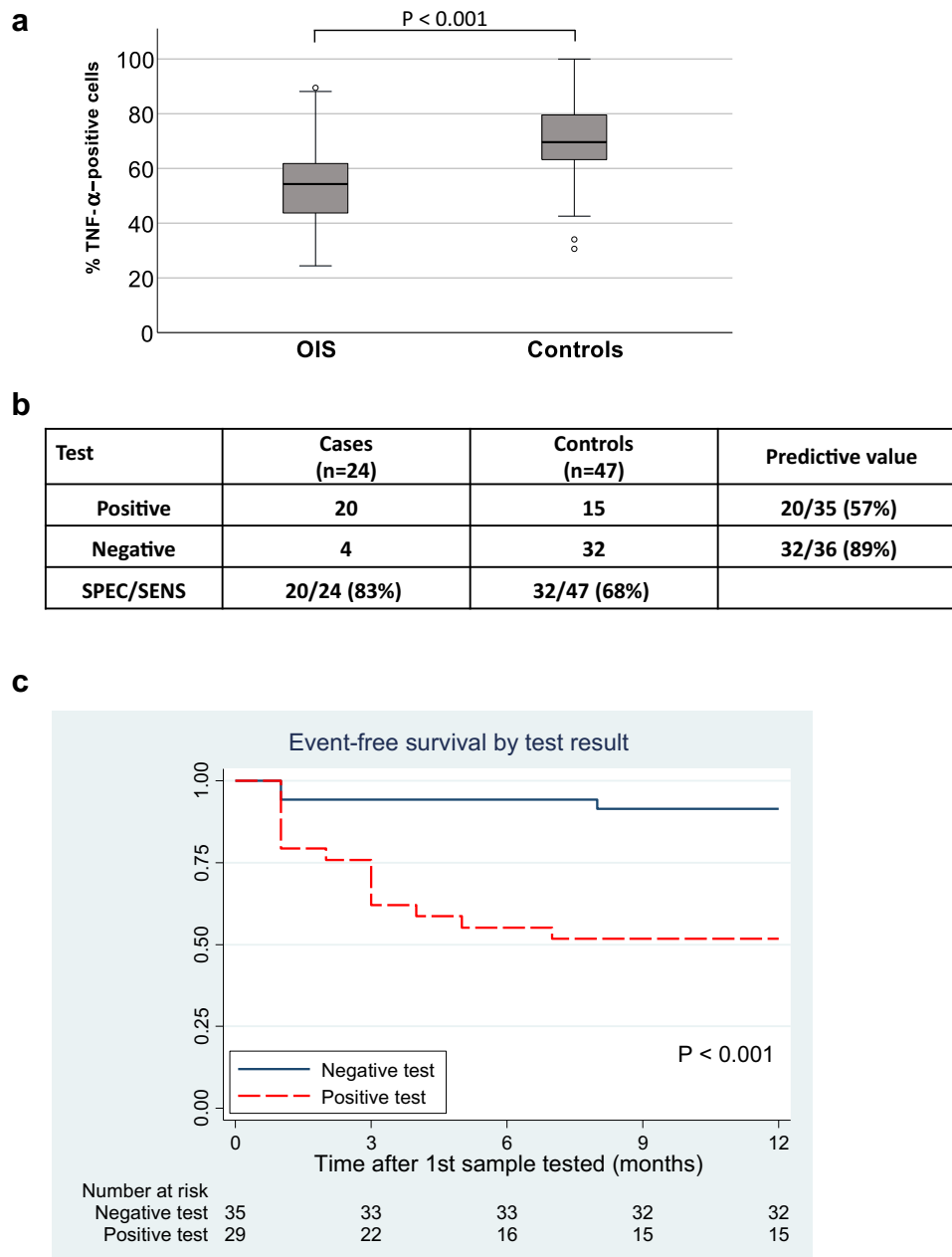### Study Population, Baseline Characteristics, and Clinical Events

Of the 73 adult kidney graft recipients who participated in the transplant biobank between January 2015 and August 2017, 71 presented viable PBMCs and were further examined as indicated in Figure 1. Characteristics of the population are presented in Table 1. Overall, patients were mostly white men and first recipients of a deceased donor. Compared with controls, OIS patients exhibited a shorter time posttransplantation, were taking higher doses of prednisone, and had higher tacrolimus serum trough levels. The proportion of patients who received induction with anti-thymocyte globulin or basiliximab was similar between groups.

Patients were classified according to their clinical phenotype by a committee blinded to the test result. OIS was defined by the occurrence of at least one of the following: an opportunistic infection, recurring infection (≥3 episodes in 12 months) in the absence of a predisposing factor, or de novo cancer. Overall, 24 patients (34%) fulfilled at least one of the OIS criteria. OIS events are listed in Table 2. The most common event

Table 2. Over-immunosuppression events in the cohort

Combination of criteria	Number of patients (n = 24)
Opportunistic infection only	14 (58)
Opportunistic and recurrent infections	5 (21)
Recurrent infections only	2 (8)
Opportunistic, recurrent infections, and de novo neoplasia	2 (8)
Opportunistic infection and de novo neoplasia	1 (4)

Data are expressed as n (%). Opportunistic infection included BK virus, John Cunningham virus, secondary cytomegalovirus, pneumocystis and cryptococcal pneumonia, oropharyngeal candidiasis, and disseminated herpes zoster. Recurrent infection was defined as ≥3 infections within 12 months in the absence of a predisposing factor.

a

b

Test	Cases (n=24)	Controls (n=47)	Predictive value
Positive	20	15	20/35 (57%)
Negative	4	32	32/36 (89%)
SPEC/SENS	20/24 (83%)	32/47 (68%)	

c

Number at risk					
Negative test	35	33	33	32	32
Positive test	29	22	16	15	15

Figure 2. CD14$^+$CD16$^+$ monocyte response. (a) Boxplots indicating the percentage of CD14$^+$CD16$^+$ monocytes producing tumor necrosis factor (TNF)–α in over-immunosuppressed (OIS) patients ($n = 24$) and controls ($n = 47$). For each patient, the percentage was computed as the mean of values at months 0 and 3 (Mann-Whitney test). (b) Diagnostic characteristics of the test. (c) Kaplan-Meier plot of event-free survival by test result (log rank test). SENS, sensitivity; SPEC, specificity.

was positive BK viremia. The first OIS event occurred at 3.6 ± 2.4 months after blood collection.

Monocyte Response to EBV Peptides

Figure 2a shows the monocyte response (mean of months 0 and 3 for each patient) according to clinical status. The percentage of TNF-α–positive CD14$^+$CD16$^+$ monocytes was lower in OIS patients than in controls (median [25–75th percentiles] 54 [44–62] vs. 70 [63–81], $P < 0.001$). Within-patient analysis indicated no significant difference in the percentage of TNF-α–positive cells between months 0 and 3, neither in the total

population (Wilcoxon signed-rank test, $P = 0.97$) nor in each group taken separately ($P = 0.93$ for OIS and $P = 0.97$ for controls). Individual data are presented in Supplementary Figure S2.

Validation of Test Accuracy, Classification Rule, and Cutoff

As displayed in Figure 2b, 20 of the 24 cases were correctly classified as having a positive test, whereas 32 of the 47 controls were correctly classified as negative. Overall, this cohort was defined by the following diagnostic accuracy: sensitivity 83% (20 of 24),

specificity 68% (32 of 47), positive predictive value (PPV) 57% (20 of 35), and negative predictive value (NPV) 89% (32 of 36). Twenty-one patients presented discordant results between samples taken at months 0 and 3, testing above the threshold at one time and below the threshold at the other. During assay development, the results of the training set suggested that these patients should be classified as negative. As 19 of them had no event, NPV was 90% (19 of 21), thus validating the classification rule in these cases.

Risk of OIS Events in Patients With Low Monocyte Response

From an immunological standpoint, OIS is a state in which the immune response is insufficient to prevent adverse events, such as opportunistic infections. Therefore, the assay can be seen as having both a diagnostic value, identifying the current OIS state, and a prognostic value, predicting the events due to this OIS state. In the present cohort, 17 of the 24 OIS patients developed the disease during follow-up, whereas 7 met the definition at the time of their first blood collection. To examine the prognostic value of the assay, we analyzed these 17 patients along with the 47 controls and estimated the risk of developing an OIS event *de novo* following a positive test. As revealed by the Kaplan-Meier curve in Figure 2c, an OIS event associated strongly with a positive test, particularly in the first 6 months following such test. The Cox proportional hazards model revealed that the risk of an OIS event was 6.8-fold higher among patients who tested positive (95% confidence interval 2.0–23.9, $P = 0.003$).

Impact of Immunosuppressive Regimen, Renal Function, Age, and Time Posttransplant on the Assay

The low number of events provided limited power to use survival models in the adjusted analysis. To test whether the relationship between the $CD14^+CD16^+$ monocyte response and the clinical status was confounded by factors known to dampen the immune response, we conducted multivariate linear regression models, in which we used the percentage of positive $CD14^+CD16^+$ monocytes as the dependent variable. The standard maintenance regimen was mostly a combination of prednisone, tacrolimus, and mycophenolate. Tacrolimus exposure was measured by trough blood levels, whereas prednisone, mycophenolate, and azathioprine exposure were measured by the prescribed dose. Consistent with the previous analysis, the unadjusted model showed that OIS was associated with significantly fewer TNF-α–positive cells (β ± SE of the mean, $-15.2\% \pm 4.0\%$, $P < 0.001$; Table 3). This association was robust also when adjusted for induction

Table 3. Univariable and multivariable estimates of the association between the percentage of TNF-α–positive $CD14^+CD16^+$ monocytes and OIS status

Unadjusted	β (% TNF-α+)	SEM	P value
Cases (ref = controls)	−15.2	4.0	<0.001

Adjustment No. 1	β (% TNF-α+)	SEM	P value
Cases (ref = controls)	−11.8	4.3	0.008
Induction			
ATG	−2.0	6.9	0.78
IL2-RI	−7.9	4.1	0.06
Prednisone, mg	−1.2	0.6	0.04
Tacrolimus dosage, ng/ml	0.9	0.9	0.29
MMF dose, 1000 mg[a]	−5.2	4.0	0.20
AZA dose, mg	0.1	0.1	0.26

Adjustment No. 2	β (% TNF-α+)	SEM	P value
Cases (ref = controls)	−13.0	4.7	0.007
eGFR, ml/min per 1.73 m²	0.1	0.1	0.61
Recipient age, y	0.2	0.2	0.40
Time posttransplant, mo	−0.02	0.1	0.61
Induction			
ATG	−3.6	7.9	0.66
IL2-RI	−10.0	5.5	0.08
Prednisone, mg	−1.2	0.6	0.08
Tacrolimus dosage, ng/ml	0.9	0.9	0.30
MMF dose, 1000 mg[a]	−4.3	4.2	0.30
AZA dose, mg	0.2	0.1	0.24

ATG, anti-thymocyte globulin; AZA, azathioprine; eGFR, estimated glomerular filtration rate; IL, interleukin; MMF, mycophenolate mofetil.
[a]In MMF equivalent.
Prednisone, tacrolimus, MMF, and AZA data were adjusted for at the time of initial blood collection.

and maintenance of immunosuppression ($-11.8\% \pm 4.3\%$, $P < 0.01$; Table 3). Further adjustment for age, renal function, and time posttransplantation resulted in a similar estimate ($-13.0\% \pm 4.7\%$, $P < 0.01$; Table 3). Adjustment for solumedrol given to patients who had rejection did not modify the association (Supplementary Table S1). Taken together, these data confirm the robustness of the association between percentage of TNF-α–positive cells and OIS status.

DISCUSSION

Tailoring immunosuppressive therapy to each patient's unique immune response is one of the most important challenges in transplantation these days.[16,17] Because OIS is now a leading cause of death and adverse events posttransplantation, the development of an "immunometer" is likely to be a major breakthrough in immunotherapy.[14] In this study, we confirmed that the $CD14^+CD16^+$ monocyte response to EBV peptides in an *ex vivo* overnight culture informed on the occurrence of OIS events in the following 12 months in kidney transplant recipients. These results confirm our previous observations and the diagnostic characteristics of

the assay. Importantly, the validation conducted here used the same threshold and classifier rule developed previously. The association between intermediate monocyte TNF-α secretion and clinical status appeared independent of age, time posttransplant, renal function, immunosuppressant levels, and doses. Hence, the assay provides useful information beyond what is currently available to clinicians when deciding about the best course of immunotherapy.

It is important to underline that the data presented here meet the definition of an external validation Type 4 outlined by the TRIPOD statement.[18] Accordingly, we used a new dataset and compared observed outcomes to the outcome predicted using the original, published classifier.[10] Because the purpose of the present work was precisely to validate previous data rather than to refine the diagnostic test, we purposely did not seek to identify a new cutoff in the current dataset. In addition, we calculated sensitivity, specificity, PPV, and NPV because their interpretation is clinically intuitive,[19] as compared with a c-statistic, which has little clinical relevance. PPV and NPV depend on prevalence of the disease in the studied population. Considering that the proportion of OIS patients in this cohort (33%, 24 of 71) is encountered also in real-life settings,[20] present PPV and NPV values are not biased by an unexpected frequency of the event. In this respect, the validated NPV is at a level similar to what led previous diagnostic tests, such as B-type natriuretic peptide, to be translated into clinical practice.[21] It is also similar to the values obtained for urinary CXCL10 and CXCL9, tested to identify rejection in the CTOT-01 trial.[22]

So far, very few markers of OIS have progressed past the development phase.[20,23,24] In particular, cell-based assays have been hampered by the fact that they are time-consuming and labor intensive. One such test, the ImmuKnow, failed to be widely adopted clinically after meta-analyses revealed limited predictive value.[25,26] One of the main advantages of the assay proposed here is that, because it measures the immune response at a single-cell level, it is not prone to bias due to cell concentration in a well, such as in enzyme-linked immunosorbent assay–based tests.[5] We have reported recently the coefficient of variation (CV) of the assay across experiments, wells, operators, and cell preparations (frozen vs. fresh). All CVs were below 5%, indicating that the assay was technically reproducible.[10] In the present case, peptides were incubated not only with monocytes but also with PBMCs. Therefore, from a biological perspective, the assay measures the capacity of the immune system as a whole to respond to foreign antigens. T-cell depletion experiments conducted while developing the assay confirmed that T cells were necessary to trigger the monocytic response.[10] Because

the readout is performed on a standard flow cytometer available in routine histocompatibility or hematology hospital laboratories, translation of the process to clinical practice should be straightforward.

There are some limitations to this study. First, OIS is not a clear-cut medical diagnosis but rather a state, whereby an insufficient immune reserve puts the patient at risk of developing adverse events. Although the definition of OIS events may be debatable, it represents a reality with serious medical and economic consequences that must be addressed.[14,27,28] Second, at this stage, the PPV of the test is modest. The threshold for positivity was initially selected based on its high NPV. The validation conducted here confirms that this is, indeed, a property of the assay in its current form. Additional work is under way to investigate how a 2-step assay can improve specificity. Nonetheless, the information provided by the test at this stage could, in itself, help prevent complications in immunosuppressed patients. For instance, in a patient maintained at a higher than average level of immunosuppression because of an increased risk of rejection, a negative test could be reassuring by indicating that the immune reserve is still adequate to prevent adverse events. Third, there was a difference in the time posttransplant between groups. This is because the samples used come from a biobank in which all patients undergoing a graft biopsy were invited to participate, in an unbiased manner. Potential confounding factors, including time posttransplant, were put into multivariable analyses. These adjusted analyses provided no indication that the association observed between percentage of TNF-α–positive cells and OIS status was confounded by this covariate. Importantly, the previous cohort used in the assay development was composed of de novo kidney recipients,[10] for whom time posttransplant was similar by definition, suggesting that this parameter is not a potential confounding factor.

In conclusion, this study confirms the diagnostic characteristics of the assay. Cell-based assays represent a novel strategy to monitor the immune reserve from peripheral blood samples. Because they are based on measuring a systemic response, they could be of major benefit to reduce the morbidity associated with immunotherapy, not only in transplant recipients but in any immunosuppressed patients. Current results highlight the importance of evaluating the assay in a larger multicenter cohort, notably to test its validity in the context of other immunosuppressive regimens and to verify how alterations in immunosuppression may impact the test result in a prospective cohort.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

FBB, IH, and SADS designed the study; FBB, OD, and SB carried out experiments. FBB and SADS analyzed the data; FBB, OD, and SADS drafted and revised the paper; and all authors approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)
Figure S1. Gating strategy.
Figure S2. $CD14^+CD16^+$ monocyte response.
Table S1. Multivariable estimates of the association between the percentage of TNF-α–positive $CD14^+CD16^+$ monocytes and OIS status, adjusted for solumedrol.

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