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Alloreactive T cells to Assess Acute Rejection Risk in Kidney Transplant Recipients

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Background. Memory T cells are important mediators of transplant rejection but are not routinely measured before or after kidney transplantation. The aims of this study were as follows: (1) validate whether pretransplant donor-reactive memory T cells are reliable predictors of acute rejection (AR) (2) determine whether donor-reactive memory T cells can distinguish AR from other causes of transplant dysfunction. **Methods.** Samples from 103 consecutive kidney transplant recipients (2018–2019) were obtained pretransplantation and at time of for-cause biopsy sampling within 6 mo of transplantation. The number of donor-reactive interferon gamma (IFN-γ) and interleukin (IL)-21-producing memory T cells was analyzed by enzyme-linked immunosorbent spot (ELISPOT) assay. **Results.** Of the 63 patients who underwent a biopsy, 25 had a biopsy-proven acute rejection (BPAR; 22 aTCMR and 3 aAMR), 19 had a presumed rejection, and 19 had no rejection. Receiver operating characteristic analysis showed that the pretransplant IFN-γ ELISPOT assay distinguished between patients who later developed BPAR and patients who remained rejection-free (area under the curve [AUC] 0.73; sensitivity 96% and specificity 41%). Both the IFN-γ and IL-21 assays were able to discriminate BPAR from other causes of transplant dysfunction (AUC 0.81; sensitivity 87% and specificity 76% and AUC 0.81; sensitivity 93% and specificity 68%, respectively). **Conclusions.** This study validates that a high number of donor-reactive memory T cells before transplantation is associated with the development of AR after transplantation. Furthermore, it demonstrates that the IFN-γ and IL-21 ELISPOT assays are able to discriminate between patients with AR and patients without AR at the time of biopsy sampling.(Transplantation Direct 2023;9: e1478; doi: 10.1097/TXD.0000000000001478.)

Memory T and B cells pose an immediate risk for transplant rejection when rechallenge with an antigen occurs.[1] To date, in vitro assays that allow for the sensitive and accurate detection of donor-specific memory T cell alloimmunity are not widely used in clinical practice.[2] Ideally, such an assay would be able to improve risk stratification before transplantation and support clinicians in fine-tuning the immunosuppressive treatment. The enzyme-linked immunosorbent spot (ELISPOT) assay has revealed that high numbers of donor-reactive interferon gamma (IFN-γ)–producing T cells are associated with the development of acute rejection (AR) after kidney transplantation.[3-7] We have shown this with the

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interleukin (IL)-21 ELISPOT assay.[8,9] IFN-γ and IL-21 are pro-inflammatory cytokines that play an active role in transplant rejection by, among others, promoting the expansion of $CD8^+$ cells and the immune responses of B cells.[10-14] AR has been associated with high numbers of donor-reactive IFN-γ and IL-21 memory T cells before transplantation or at 3–6 mo after transplantation.[8,9,15-18] However, the dynamics of donor-reactive cells during the process of AR remains unknown. By measuring donor-reactive cells at the time of biopsy sampling, we may be able to correlate these cell numbers in the peripheral blood to specific clinical events. This study aimed to (1) investigate whether donor-reactive cells in peripheral blood can be used to discriminate patients with an AR from patients with other causes of kidney dysfunction and (2) validate whether donor-reactive cells can be used to identify kidney transplant recipients at risk for AR.

MATERIALS AND METHODS

Patients and Samples

Adult kidney transplant candidates who were admitted to receive a kidney transplant at the Erasmus MC, University Medical Center Rotterdam between August 2018 to May 2019 participated in the Liquid Biopsies for minimally invasive Detection of AR study which aims to identify minimally invasive biomarkers for the diagnosis of acute kidney transplant rejection.[19,20] All patients provided written informed consent and the study was approved by the Medical Ethics Committee of the Erasmus MC Rotterdam, the Netherlands (number 2010-022 and number 2018-035). All experiments were performed in accordance with the relevant guidelines and regulations of our institution and in accordance with the ethical standards of the Declaration of Helsinki. The postoperative immunosuppressive regimen after transplantation consisted of tacrolimus (Prograf; Astellas Pharma, Tokyo, Japan; aiming for predose concentrations of 10–15 ng/mL in weeks 1–2, 8–12 ng/mL in weeks 3–4 and 5–10 ng/mL thereafter), mycophenolate mofetil (Cellcept); Roche, Basel, Switzerland; starting dose of 1 g twice a day, aiming for predose concentrations of 1.5–3.0 mg/L) and prednisolone. Prednisolone was tapered to 5 mg at month 3 and withdrawn at months 4–5.

Whole blood samples were collected both before transplantation and within 48 h before a for-cause kidney transplant biopsy. All patients were followed up until 6 mo posttransplantation. For-cause biopsies were classified by a nephropathologist (MCvG) according to the Banff'19 update,[21] **Table S1, SDC,** http://links.lww.com/TXD/A527. Patients were classified into a "biopsy-proven acute rejection (BPAR)" group (having at least one BPAR), a "presumed rejection" group (patients without histologically confirmed rejection but treated with anti-rejection therapy) and a "no rejection" group (biopsies demonstrating an alternative diagnosis to rejection, that is, acute tubular necrosis [ATN] or no biopsy). Biopsy samples within the BPAR group were classified as "aAMR" or "aTCMR." Biopsy samples in the "presumed rejection" group were classified as "suspicious for AMR" (ie, histological evidence of AMR such as microvascular inflammation or thrombotic microangiopathy but without C4d positivity or donor-specific antibodies [DSA]), biopsies with clinically suspected rejection (no histologic evidence of rejection but with a favorable response to anti-rejection therapy) and biopsies with borderline histological lesions for TCMR. The "no rejection" group consisted of biopsy samples with ATN or histopathological lesions other than rejection. In recipients with multiple biopsies, the first biopsy sample was used for analysis. The second biopsy sample was only used for analysis whenever blood collection was missed at time of first biopsy.

Detection of Anti-HLA Antibodies

Serum samples were screened for the presence of anti-HLA antibodies using the Lifecodes Lifescreen Deluxe kit (Immucor Transplant Diagnostics Inc. software [Stamford, CT]. Anti-HLA class I or anti-HLA class II antibodies were analyzed with a Luminex Single Antigen assay using LABscreen HLA class I and II antigen beads (One Lambda, Canoga Park, GA). The data were analyzed using the MATCHIT! antibody software version 1.3.1 (Immucor). The results were expressed as raw or background-corrected mean fluorescence intensity (MFI). A bead-specific cut-off based on raw MFI/lowest-ranked antigen, in combination with raw MFI >750, was used to assign positive beads. The presence of DSA was determined by comparing the measured HLA specificities with donor HLA typing.

Peripheral Blood Mononuclear Cells and Spleen Cells

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from heparinized blood by use of standard Ficoll separation (GE Healthcare, Uppsala, Sweden) and stored at −140°C until use. Donor spleen cells were obtained by mechanical dissociation of spleen from the organ donor. After filtration of the cell suspension, the cells were isolated by Ficoll separation.

IFN-γ and IL-21 ELISPOT Assay

ELISPOT assays were performed as described in a previous study.[9] In brief, patient PBMCs were incubated on plates coated with anti-human IFN-γ or IL-21 mAb (U-CyTech Biosciences, Utrecht, the Netherlands) with irradiated (40 Gy) PBMCs or spleen cells derived from the donor or irradiated third-party cells, which were completely HLA-mismatched with donor and recipient to determine an overall HLA response. The incubation period of the IFN-γ and IL-21 assays was 20 h and 44 h, respectively. The number of cells per sample seeded in a well was 1×10^5 cells/well and 3×10^5 cells/well for the IFN-γ and IL-21 assay, respectively. Cells were incubated stepwise with biotinylated anti-human IFN-γ or IL-21 detection antibody (U-CyTech Biosciences), streptavidin–HRP conjugate (U-CyTech Biosciences), and AEC substrate (U-CyTech Biosciences) according to manufacturer instructions until distinct spots formed. Spots were counted automatically by using a Bioreader 6000 ELISPOT reader (Bio-Sys GmbH, Karben, Germany).

Statistical Analysis

Statistical analyses were performed using SPSS 21.0 (SPSS Inc, Chicago, IL). Figures were made with GraphPad Prism version 6.01 (GraphPad, Inc., La Jolla, CA). The Kruskal-Wallis test with Bonferroni correction for multiple comparisons was used for comparisons of donor-reactive cells between patients with no rejection, presumed rejection, and BPAR. Data are presented as median and interquartile range, **Table S2, SDC,** http://links.lww.com/TXD/A527. Pearson's correlations were performed to determine the strength between IFN-γ and IL-21 donor-reactive memory T cells. Receiver operating characteristic (ROC) curve analysis was used to calculate the cut-off value of number of donor-reactive cells. The threshold value of donor-reactive cells was selected by a data-driven approach

using the Youden's index. ROC curve analysis was used to calculate the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the ELISPOT assay. Multivariable binary logistic regression was performed to assess the odds ratio (OR) and 95% confidence interval (CI) for developing a BPAR. The regression was done using a stepwise backward selection method. A 2-sided $P \leq 0.05$ was considered statistically significant. Finally, the degree of donor reactivity before transplantation and at the time of biopsy sampling were compared using the chi-squared test.

RESULTS

Patients

Table 1 depicts the baseline characteristics of the 103 transplant recipients included in this study. The median age at the time of transplantation was 61.4 y and most patients received their first kidney transplant (84%). Approximately half (54%) of the patients received a kidney from a deceased donor. Three patients had detectable DSA before transplantation, 1 patient was treated with alemtuzumab, IVIg, and plasma exchange, the second patient was treated with anti-thymocyte globulin, IVIg, plasma exchange, and rituximab and the third patient had a negative crossmatch test and was treated with basiliximab (standard induction agent in our center). A total of 97 for-cause biopsies were performed within 6 mo after transplantation in 63 patients. The histopathologic findings are described in **Table S1, SDC,** http://links.lww.com/TXD/A527. Of the 63 patients, 25 (24.3%, one of which had pretransplant DSA) were classified as having "BPAR," 19 (18.4%, 2 had pretransplant DSA) were classified as having a "presumed rejection," whereas 19 patients (18.4%, 17 ATN, 1 aTMA, and 1 diabetic nephropathy) were classified as "no rejection."

TABLE 1.

Patient characteristics

Recipient and donor characteristics study population	Study population (n = 103)
Age, y	61.4 (12.7)
Sex	
Female/male	40 (39%)/63 (61%)
Ethnicity	
Caucasian	79 (77%)
African	14 (13%)
Asian	6 (6%)
North African/Arab	3 (3%)
Other	1 (1%)
Primary kidney disease	
Diabetic nephropathy	22 (21%)
Hypertensive nephropathy	22 (21%)
Glomerulonephritis	18 (17%)
Polycystic kidney disease	10 (10%)
Unknown	5 (5%)
Reflux nephropathy/chronic pyelonephritis	1 (1%)
Other	25 (25%)
Total number of transplantations	
1	87 (84%)
2-3	16 (16%)
Donor type	
DBD	23 (22%)
DCD	33 (32%)
Living donor-related	15 (15%)
Living donor-unrelated	32 (31%)
Donor sex	
Female/male	49 (48%)/54 (52%)
Total mismatch number	
0	6 (6%)
1-2	30 (29%)
3-4	48 (47%)
5-6	19 (18%)
Induction therapy	
Basiliximab	98 (95%)
Alemtuzumab	2 (2%)
Alemtuzumab, IVIG, and plasmapheresis	1 (1%)
ATG, IVIG, plasmapheresis, and rituximab	1 (1%)
None	1 (1%)

Continuous variables are described as mean (SD). Categorical variables as number of cases (%). ATG, anti-thymocyte globulin; DBD, donation after brain death; DCD, donation after circulatory death.

Pretransplant Donor-reactive Cells and Development of BPAR

A three-way comparison of patients with BPAR, presumed rejection, and no rejection showed that the number of donor-reactive IFN-γ producing cells was significantly different between the groups ($P = 0.002$). Subgroup analysis demonstrated that patients who developed BPAR had significantly higher numbers of donor-reactive IFN-γ cells compared with patients who did not (57 [35–82] versus 31 [14–48]; $P = 0.006$, Figure 1A). In the case of IL-21 producing donor-reactive cells, there was no significant difference in donor-reactive IL-21 producing cells ($P = 0.06$, Figure 1C). There was no difference in third-party–reactive IFN-γ and IL-21 producing cells in patients with or without a BPAR (Figure 1B and 1D). When patients with DSA were excluded, the results remained consistent with the results of the analyses including patients with DSA. Pearson's correlation was used to assess the relationship between the number of donor-reactive IFN-γ and IL-21 producing cells. There was a positive correlation between these 2 variables with a correlation coefficient of r = 0.38 ($P < 0.001$; 95% CI, 0.19-0.54).

The diagnostic accuracy of the IFN-γ ELISPOT assay was assessed in a ROC curve analysis. A cut-off value of 20 donor-reactive IFN-γ producing cells per 1×10^5 PBMC resulted in an area under the curve (AUC) of 0.73 and was able to discriminate patients with BPAR within 6 mo posttransplantation from patients without rejection with a sensitivity of 96% and specificity of 41% (Figure 1E). Based on the rate of BPAR (24.3%), this cut-off resulted in a PPV of 34% and NPV of 97%.

Furthermore, binary logistic regressions showed that the highest historical PRA (OR = 1.02; 95% CI, 1.00-1.04; $P = 0.01$) and positive pretransplant IFN-γ assay (OR = 15.11; 95% CI, 1.91-119.79; $P = 0.01$) were significant predictors for the development of BPAR within 6 mo after transplantation (**Table S3, SDC,** http://links.lww.com/TXD/A527).

Donor-reactive Cells at the Time of Biopsy Sampling

To determine whether donor-reactive cells can be used to distinguish patients with an AR from patients with other causes of kidney dysfunction, these cells were measured at time of biopsy. The diagnoses of the first biopsy sample were classified as 19 BPAR (16 aTCMR and 3 aAMR), 20 presumed rejections (of which 6 were suspicious for aAMR),

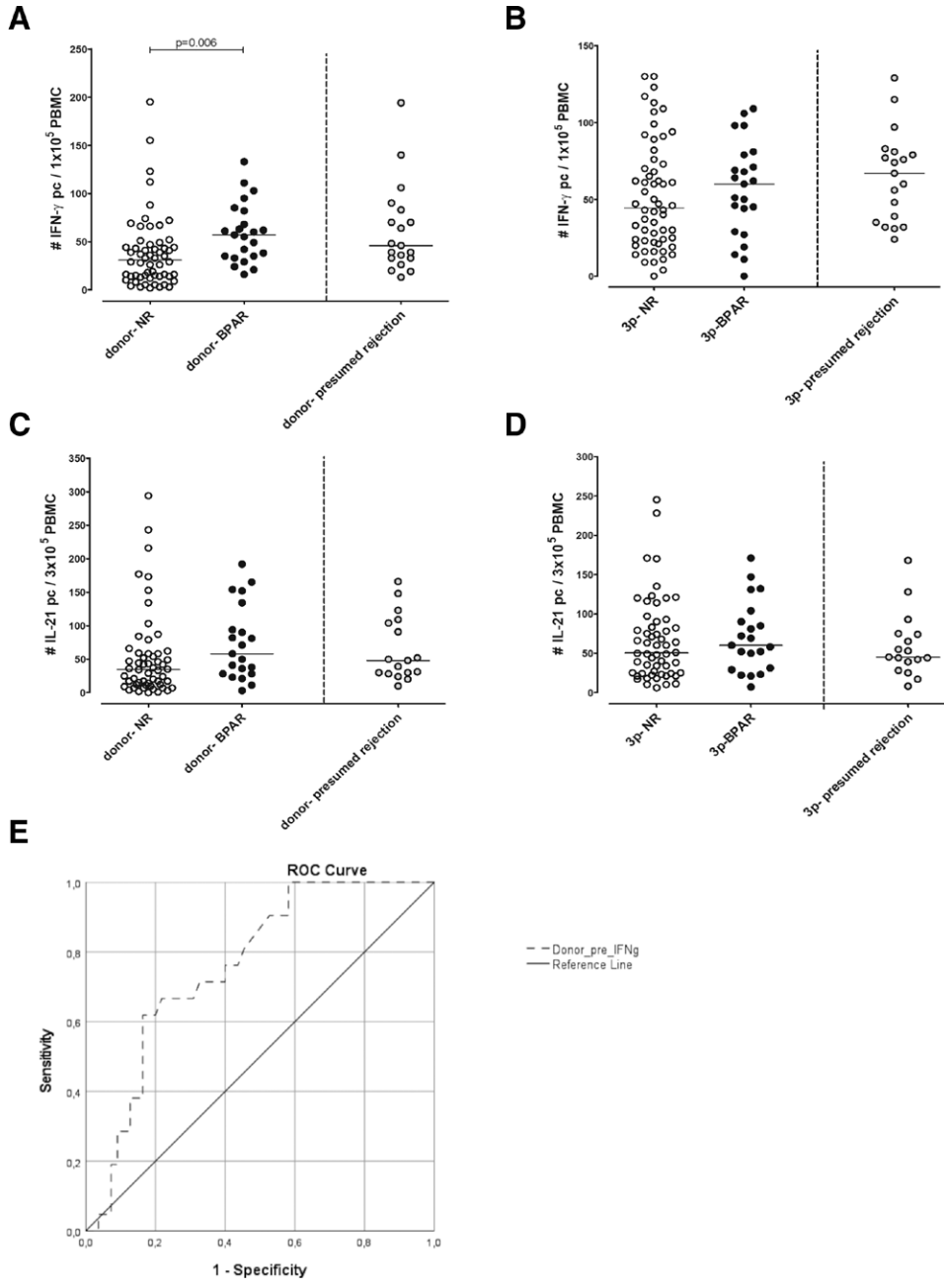

FIGURE 1. Number of pretransplant donor-reactive (A) IFN-γ– and (C) IL-21–producing cells, third-party–reactive (B) IFN-γ and (D) IL-21 in patients with no rejection, presumed rejection, and BPAR and (E) ROC curve of donor-reactive IFN-γ enzyme-linked immunosorbent spot assays to evaluate its performance in predicting BPAR within 6 mo after transplantation. Because of limited sample materials, n = 100 (IFN-γ) and n = 94 (IL-21) samples were included in analysis. BPAR, biopsy-proven acute rejection; IFN, interferon; IL, interleukin; NR, no rejection; PBMC, peripheral blood mononuclear cells; ROC, receiver operating characteristic.

and 24 no rejection (22 ATN, 1 aTMA, and 1 diabetic nephropathy). In a three-way analysis, the patients at time of BPAR had a significantly higher number of donor-reactive IFN-γ producing cells compared with patients with no rejection ($P < 0.001$). However, the number of donor-reactive IFN-γ producing cells in patients with BPAR and presumed rejection was not significantly different (Figure 2A). A three-way analysis comparing patients during BPAR, presumed rejection, and no rejection showed that the number of IL-21 donor-reactive cells was significantly different between the three groups ($P = 0.008$). In a subgroup analysis, patients during BPAR had higher numbers of donor-reactive cells than patients with no rejection ($P = 0.003$, Figure 2C). There was also a higher number of IL-21 producing donor-reactive cells in patients with BPAR when compared with patients with a presumed rejection (56 [42–118] versus 27 [21–47]; $P = 0.02$). There was no significant difference in third-party–reactive IFN-γ and IL-21 producing cells in patients with or without BPAR (Figure 2B and 2D). Because of the small number of patients with a diagnosis of aAMR, it was not possible to perform subgroup analysis based on the type of rejection (**Figure S1, SDC,** http://links.lww.com/TXD/A527). At the time of biopsy, there was a positive correlation between the number of IFN-γ and IL-21 producing donor-reactive cells

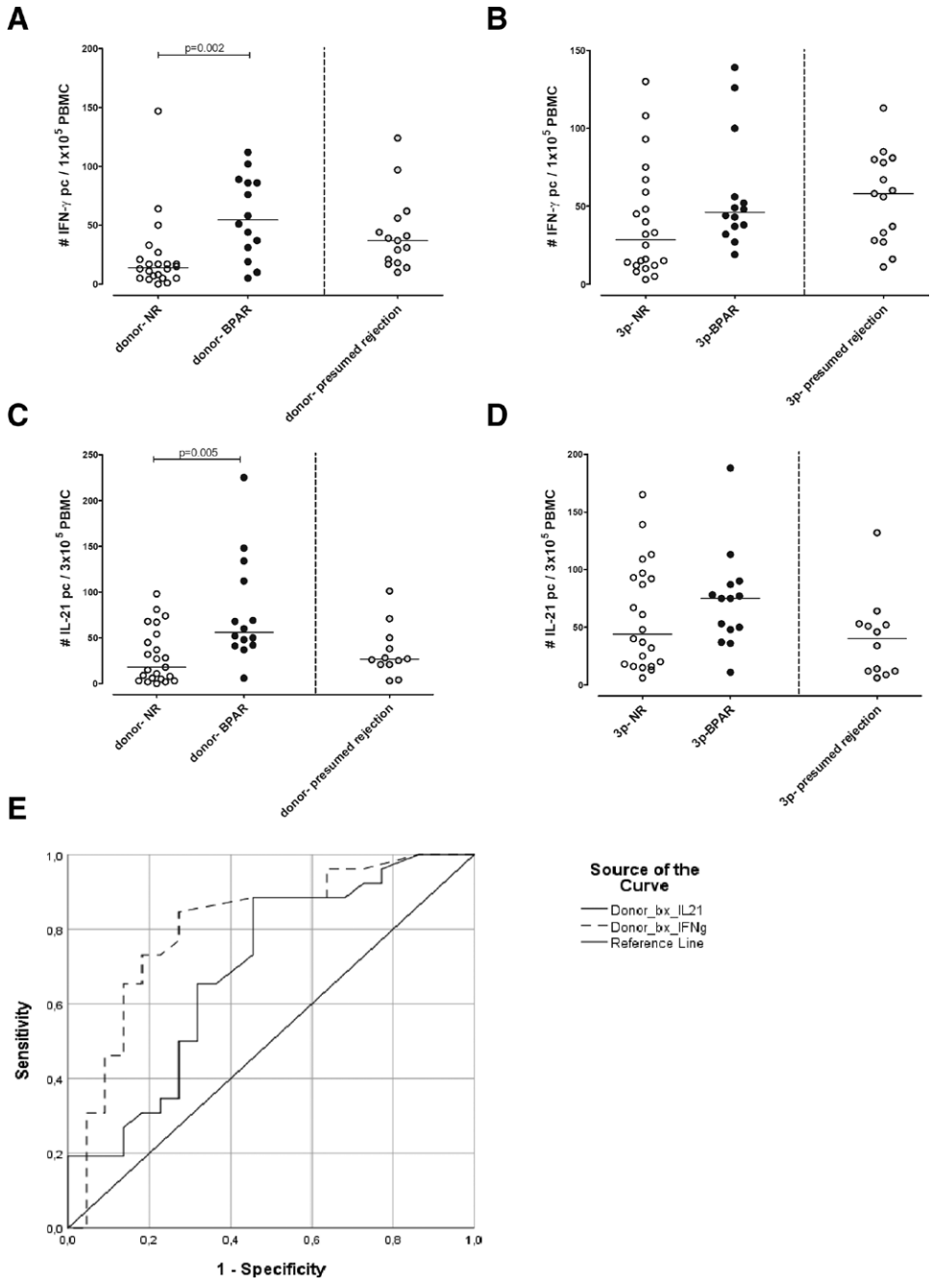

FIGURE 2. Number of donor-reactive (A) IFN-γ– and (C) IL-21–producing cells, third-party–reactive (B) IFN-γ and (D) IL-21 in patients with no rejection, presumed rejection, and BPAR at the time of biopsy sampling and (E) receiver operating characteristic curve of donor-reactive IFN-γ and IL-21 enzyme-linked immunosorbent spot assays to evaluate their performance in identifying patients with BPAR at the time of biopsy sampling. Because of limited sample materials, n = 51 (IFN-γ) and n = 48 (IL-21) samples were included in analysis. BPAR, biopsy-proven acute rejection; IFN, interferon; IL, interleukin; NR, no rejection; PBMC, peripheral blood mononuclear cells.

with a correlation coefficient of r = 0.38 (P = 0.009; 95% CI, 0.10-0.60).

ROC analysis showed that a cut-off value of 18 donor-reactive IFN-γ producing cells per 1×10^5 PBMC resulted in an AUC of 0.81 and was able to discriminate patients during early rejection with a sensitivity of 87% and specificity of 76% (Figure 2E). Based on the rate of BPAR (19/103 = 18.4%), this cut-off resulted in a PPV of 45% and NPV of 96%. Finally, a cut-off value of 34 donor-reactive IL-21 producing cells per 3×10^5 PBMC resulted in an AUC of 0.81 and was able to discriminate patients during early rejection with a sensitivity of 93% and specificity of 68% (Figure 2E). This resulted in a PPV of 40% and NPV of 98%.

Finally, we found that patients with a high degree of pretransplant IFN-γ donor-reactive cells (defined as an ELISPOT assay above the respective cut-off value) retained a high donor-reactivity at the time of biopsy (26/39 [66.7%]; P = 0.003, **Table S4, SDC,** http://links.lww.com/TXD/A527). Consequently, patients who had a low number of pretransplant IFN-γ donor-reactive cells (defined as an ELISPOT assay below the respective cut-off value) also had lower donor-reactivity at the time of biopsy (10/12 [83.3%]).

DISCUSSION

The aims of this study were to determine whether donor-reactive cells can discriminate patients with an AR from patients with other causes of kidney dysfunction and to validate whether donor-reactive cells can reliably determine the risk of rejection. We show that donor-reactive cells are highest in patients who have a BPAR. The fact that donor-reactive cells are associated with BPAR (Figure 2) means that these cells are likely directly involved in the process of AR. The ELISPOT assay has mainly been used as a tool for pretransplant screening, but it is not clear whether memory alloreactive T cells are elevated in the peripheral blood during BPAR. Alachkar et al (2016),[22] found an expansion of the T-cell receptor repertoire and higher $CD4^+$ and $CD8^+$ RNA expression levels in blood samples during rejection. A high number of IFN-γ donor-reactive cells was already present in the pretransplant sample of approximately two-thirds of kidney transplant recipients. This signifies that in most patients these cells persist after transplantation despite the use of immunosuppressive therapy. Alternative induction therapy or a personalized immunosuppressive regimen might be necessary to prevent AR in patients with high anti-donor reactivity. Patients with high anti-donor reactivity might benefit from T cell depleting induction therapy, such as anti-thymocyte globulin, over the use of the more commonly used monoclonal antibody basiliximab. Alternatively, patients with a low anti-donor reactivity are likely good candidates for a reduction in the immunosuppressive load. This may involve the use of tacrolimus monotherapy or the use of lower target ranges of either tacrolimus or mycophenolate mofetil therapy. Currently, there is one randomized control trial (CELLIMINI trial) that has found that patients with low T cell donor-reactivity in combination with lower class II HLA eplet mismatch can be safely treated with tacrolimus monotherapy without an increase in AR rates.[23]

In this cohort of consecutive kidney transplant recipients, we confirmed that the number of donor-reactive IFN-γ producing cells was higher in patients who developed a BPAR within 6 mo after transplantation compared with patients who did not. Our findings are consistent with a meta-analysis of the ELISPOT assay, where the authors demonstrated that the IFN-γ assay is reliable in predicting AR in kidney transplant recipients.[24]

Pretransplant screening for the presence of anti-HLA antibodies is routine practice in patients waiting for kidney transplantation.[25-27] Although screening for anti-HLA antibodies has significantly decreased the incidence of hyperacute AMR,[28] it fails to identify adaptive anti-HLA specific memory T cell responses. The findings of this study support the implementation of pretransplant screening of donor-reactive memory T cells to assess the cellular immunity of individual patients and their risk to develop aTCMR.

Because of the minimally invasive nature of the ELISPOT assay, it can also be used to monitor the immune state of patients after kidney transplantation. Many studies have investigated the immune responses of T and B cell subsets, cytokines (eg, CXCL10, CXCL10, and IFN-γ), transcriptomics, proteomics, and donor-derived cell-free DNA with the aim of finding alternative markers for transplant rejection.[29] Despite the large amount of research on noninvasive biomarkers,[30] none of these markers have been systematically applied in current clinical practice. This may be because of several reasons including, but not limited to challenges in the reproducibility of single-center studies and a lack of biomarkers with both high sensitivity and specificity for the detection of AR. The high NPV of the ELISPOT assay makes it a particularly useful technique to rule out AR, as a low number of donor-reactive cells means that the probability of AR is extremely low. Posttransplant use of the ELISPOT assay can potentially decrease the need for biopsy sampling in patients with low anti-donor reactivity. Because of the low PPV of the ELISPOT assay, a combination of minimally invasive biomarkers would likely be necessary to improve the diagnostic sensitivity to detect BPAR.[31,32] There is evidence that a combination of several biomarkers is likely to improve the diagnostic utility of a biomarker compared with the use of a single biomarker.[32] Both the IFN-γ and IL-21 ELISPOT assays measure a memory T cell response from peripheral blood samples. The use of mechanistically distinct biomarkers, for example, urine markers in combination with the ELISPOT assay, is worth exploring for more accurate risk profiling or diagnosis of rejection.

Future studies should aim to understand the dynamics of donor-reactive cells after transplantation. It would be interesting to know whether high numbers of donor-reactive cells are only present during episodes of AR or whether certain patient populations have a continuously high anti-donor response. It would be interesting to see whether serial monitoring of donor-reactive cells every 1–3 mo in the first year after transplantation can lead to the detection of subclinical AR. It is also necessary to investigate whether the number of donor-reactive cells can be reduced with the use of anti-rejection therapies (eg, high-dose pulse methylprednisolone or alemtuzumab) and whether significant reduction of these cells can prevent future episodes of AR. Patients with high alloreactivity at all times may require more intense immunosuppression than patients with low alloreactivity. Additionally, if donor-reactive cells are more dynamic and are shown to specifically increase during episodes of AR, the value of the ELISPOT assay as a biomarker for AR becomes much greater.

The findings of this article should be viewed in light of the study's limitations. Firstly, the data presented in this study are single-center results and they should be further validated in a multicenter study. The ELISPOT assay requires the availability of viable donor cells which, because of logistics, can be challenging, especially in the case of deceased kidney donation. Finally, the ELISPOT assay involves long incubation periods (IFN-γ 20 h, IL-21 44 h) that makes this assay better suited for pretransplant screening in patients receiving a kidney transplant from a living donor. This does not discount the use of the assay in patients who receive a deceased kidney donor; however, the assay can still be used to create a risk profile for the development of AR.

In conclusion, a high pretransplant number of donor-reactive memory T cells is associated with the subsequent development of AR. Second, donor-reactive memory T cells are elevated at the time of BPAR.

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