Circulating B Cells With Memory and Antibody-Secreting Phenotypes Are Detectable in Pediatric Kidney Transplant Recipients Before the Development of Antibody-Mediated Rejection

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Background. Development of anti–human leukocyte antigen donor-specific antibodies (DSAs) is associated with antibody-mediated rejection (AMR) and reduced allograft survival in kidney transplant recipients. Whether changes in circulating lymphocytes anticipate DSA or AMR development is unclear. Methods. We used time-of-flight mass cytometry to analyze prospectively collected peripheral blood mononuclear cells (PBMC) from pediatric kidney transplant recipients who developed DSA (DSA-positive recipients [DSAPOS], n = 10). PBMC were obtained at 2 months posttransplant, 3 months before DSA development, and at DSA detection. PBMC collected at the same time points posttransplant from recipients who did not develop DSA (DSA-negative recipients [DSANEG], n = 11) were used as controls. Results. DSAPOS and DSANEG recipients had similar baseline characteristics and comparable frequencies of total B and T cells. Within DSAPOS recipients, there was no difference in DSA levels (mean fluorescence intensity [MFI]: 13687 ± 4159 vs 11375 ± 1894 in DSAPOSAMR-positive recipients (AMRPOS) vs DSAPOSAMR-negative recipients (AMRNEG), respectively; P = 0.630), C1q binding (5 DSAPOSAMRPOS [100%] vs 4 DSANEGAMRNEG [80%]; P = 1.000), or C3d binding (3 DSAPOSAMRPOS [60%] vs 1 DSANEGAMRNEG [20%]; P = 0.520) between patients who developed AMR and those who did not. However, DSAPOS patients who developed AMR (n = 5; 18.0 ± 3.6 mo post-DSA detection) had increased B cells with antibody-secreting (IgD−CD27+CD38+; P = 0.003) phenotypes compared with DSANEG and DSAPOSAMRNEG recipients at DSA detection. Conclusions. Despite the small sample size, our comprehensive phenotypic analyses show that circulating B cells with memory and antibody-secreting phenotypes are present at DSA onset, >1 year before biopsy-proven AMR in pediatric kidney transplant recipients.

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Short-term kidney transplantation outcomes have improved significantly over the past decades with the implementation of induction therapies and calcineurin inhibitor (CNI)-based immunosuppression regimens. While these treatments reduce episodes of acute cellular rejection, they have failed to improve long-term allograft survival, with only 50%-60% of allografts functioning after 10 years. The reasons for long-term allograft failure are multifactorial, but development of de novo donor-specific anti-human leukocyte antigen (HLA) antibodies (dnDSAs) is recognized as a leading cause, affecting up to 30% of unsensitized kidney transplant recipients, with 1%-10% occurring within the first year posttransplant.

DSA-positive recipients (DSAPOS) are at increased risk of antibody-mediated rejection (AMR), a condition that can lead to accelerated allograft failure and for which treatment strategies are still not standardized. Highly sensitized patients with pretransplant DSA incur a substantially higher rate of AMR than their DSA-negative counterparts. However, predicting which unsensitized recipients will develop dnDSA, and of those which will suffer AMR, remains difficult. Recent studies suggest that the ability of DSA to activate the complement cascade, assessed via C1q- or C3d-binding assays, correlates with allograft loss and can help risk-stratify DSAPOS recipients. However, data about the utility of these measures in clinical practice have not been consistent thus far.

Memory B cells are formed within germinal centers following the primary encounter with alloantigen and are able to generate an accelerated immune response upon antigen re-encounter. Memory B cells are also detectable in the peripheral blood of highly sensitized recipients before and during an AMR episode, even in the absence of circulating DSA. However, no study to date has comprehensively looked at the immune phenotype of immunologically naive transplant recipients to investigate whether other immunologic perturbations precede antibody development or AMR.

One reason for the lack of comprehensive immune phenotyping of transplant patients is that standard flow cytometry is limited in the number of markers that can be probed in a single experiment due to autofluorescence and spectral overlap associated with fluorophores. Time-of-flight mass cytometry (CyTOF) utilizes metal isotopes that possess unique mass spectrometry signatures enabling the analysis of up to 50 cellular markers at the same time. Furthermore, CyTOF reduces experimental variability as metal isotopes can be used to tag samples with barcodes, allowing multiple samples to be analyzed simultaneously. We used CyTOF to test the hypothesis that changes occur in the phenotype of circulating T and/or B cells before the development of DSA or AMR. To do this, we comprehensively analyzed immune phenotypes of prospectively collected peripheral blood mononuclear cells (PBMC) from pediatric kidney transplant recipients who did or did not develop dnDSA, with or without AMR.

**Materials and Methods**

**Subjects and Sample Collection**

Pediatric subjects (<18 y at the time of transplant) transplanted at Gaslini Hospital in Genoa, Italy, between August 2003 and March 2013 underwent serial measurement of circulating DSA at months 1, 2, 6, 9, 12 posttransplant, and every 6 months thereafter. At the time of each DSA measurement, patients also had PBMC collected and stored in liquid nitrogen. During the study period, 136 kidney transplants were consecutively performed.

Patients were included in this study if they were recipients of a first kidney graft and nonsensitized (Panel-reactive antibody = 0; absence of any HLA antibody (Ab) in historical sera tested before kidney transplant; n = 98). We performed a case-control study, where we analyzed serially collected PBMC aliquots at 2 months posttransplant, at the last available visit before DSA development, and at the time of first DSA detection in all the consecutive patients who developed DSA within the first 12 months after transplant (n = 10) and 11 patients who received a kidney transplant during the same time period (±6 mo) at the same institution but did not develop DSA during the same follow-up time posttransplant. Demographic and clinical characteristics of donors and recipients were collected up to 6 years posttransplant. The protocol has been approved by the Gaslini Hospital Institutional Review Board (IRB 867/2014).

**Patient Treatment and Management**

All kidney transplant recipients received the same immunosuppressive therapy consisting of anti-CD25 antibody induction (basiliximab) and maintenance with mycophenolate mofetil and a CNI (either tacrolimus or cyclosporine) with or without steroids. Tacrolimus target trough levels were 8–10 ng/mL in the first month posttransplant, 7–9 ng/mL from months 2–3, and 6–8 ng/mL thereafter. Two-hour postdose target levels of cyclosporine in whole blood (C1) were 1400–1800 ng/mL in the first month posttransplant, 1200–1600 ng/mL in the second month, 1000–1400 from months 3–6, and 800–1200 thereafter (see adherence calculations in section below). Graft biopsies were performed as per clinical indication, including DSA development. Diagnosis of AMR was based on Banff 2013.

**Detection and Characterization of HLA Abs**

HLA classes I and II typing were performed as described previously. Anti-HLA classes I and II IgG Abs were tested with a bead-based detection assay. We used the LABScreen Mixed kit (One Lambda; Thermo Fisher Scientific, Waltham, MA), which simultaneously detects classes I and II Abs, and the single antigen bead assays (Single Antigen kit, One Lambda; Thermo Fisher Scientific) to identify HLA classes I and II specificities. Before testing, all sera were pretreated with disodium EDTA (final concentration 10 mM, pH 7.4; Sigma-Aldrich, Milan, Italy) to rule out underestimation of Ab mean fluorescence intensity (MFI) strength due to the prozone phenomenon. Screening assay results above a cutoff value of 3.0 for the ratio of sample to negative control were considered positive. Single-antigen results above an MFI cutoff value of 1000 were considered positive.

Heat-inactivated patient sera were tested with C1qScreen (One Lambda; Thermo Fisher Scientific) for identification of complement-binding Abs, as described. Serum samples were analyzed in a blinded fashion for the presence of C3d-binding DSAs with the single-antigen flow bead technology, according to the manufacturer’s protocol (Immucor Lifecode Transplant Diagnostics, Nijlen, Belgium).

**Adherence to CNI Therapy**

Routine laboratory measurement of 2-hour postdose cyclosporine (C1) levels and tacrolimus trough levels in whole
blood were determined using an antibody-conjugated magnetic immunoassay method and by microparticle enzyme immunoassay based on the Abbott IMx (Abbott Laboratories, Abbott Park, IL), respectively.

Tacrolimus and cyclosporine C_{2} levels were collected over a period of >1 year. Intrapatient variability (IPV) has been shown to be a useful surrogate for CNI adherence and a predictor of long-term kidney transplant outcomes.42-44 Given the small sample size of our cohort, CNI IPV was described by the coefficient of variation (CV%) and calculated as the square root of the ratio of the variance to the mean, 

\[
(CV\%) = \sqrt{\frac{\sum (X_{i} - \bar{X})^2}{n(n-1)}} / \bar{X} \times 100, \text{ which has been shown to provide an accurate estimate for these studies.}^{42}
\]

Only levels collected after 6 months posttransplant, when dosing is assumed to be stable, were included in IPV calculations.

CyTOF Sample Preparation

Our aim was to map the peripheral blood immune system of renal transplant recipients and its evolution over time, while minimizing batch effect, to increase our ability to capture differences in frequencies of immune cell compartments. To do this, we barcoded samples collected from the same recipients at the 3 different time points with anti-CD45 antibodies conjugated to unique metal isotopes before pooling the samples together. CyTOF sample preparation was conducted as previously reported.45,46 Antibodies were either purchased preconjugated from Fluidigm (formerly DVS Sciences) or purchased purified and conjugated in-house using MaxPar X8 Polymer Kits (Fluidigm) according to the manufacturer’s instructions. Sixty-three samples (21 recipients with approximately 3 time points each) were processed in 4 separate batches using 4 barcoding antibodies for each sample (to denote patient and time point) and pooled together (Figure 1). All PBMC were stained with a panel of 35 antibodies (34 for clustering, 1 for viability; see Table S1, SDC, http://links.lww.com/TXD/A217).

CyTOF Data Acquisition

CyTOF data were acquired at Icahn School of Medicine at Mount Sinai as previously reported by others.45,46 Samples were suspended in deionized water containing a 1/20 dilution of EQ 4 Element Beads (Fluidigm) at a concentration of 1 million cells/mL and acquired on a CyTOF2 (Fluidigm) equipped with a SuperSampler fluidics system (Victorian Airships) at an event rate of <500 events/s. After acquisition, the data were normalized using bead-based normalization in the CyTOF software. Barcodes were demultiplexed using the Fluidigm debarcoding software. The data were gated to exclude residual normalization beads, debris, dead cells, and doublets, leaving live CD45^+ events for subsequent clustering and high-dimensional analyses.

CyTOF Data Analysis

We utilized a CyTOF analysis pipeline that was previously established in our laboratory.46 We first clustered cells using the Phenograph algorithm47 and then curated the metaclusters obtained. The frequencies for each common population were obtained by summation of the frequencies in each metacluster and subsequently debarcoded to obtain frequencies for each time point and patient. To minimize variability in measurement, our analysis strategy was structured as follows:

1. Application of Phenograph to computationally pooled samples: we pooled in silico all labeled cells from all time points for each patient to analyze the major immune compartments (10,000 cells per time point for an average of 30,000 cells per patient); we applied Phenograph and then demultiplexed them. This allowed us to map the same subsets in all the samples, as opposed to gating each sample for each patient individually.

2. Equal contribution of the samples: to avoid bias in Phenograph for the subsets present in the samples with a greater total number of T or B cells, we sampled 500 cells per sample for the CD4^+ and CD8^+ T cell subclustering. For the B-cell compartment, not all samples had 500 cells. For the 5 recipients who had at least 1 PBMC sample with...
<500 B cells, we adjusted the contribution of cells to maximize the number of B cells analyzed. In this way, we were able to minimize clustering bias while maintaining equal contribution from each sample.

3. Reiteration: to increase power of the analyses for the cell subsets with low number of events, we reiterated the entirety of the B-cell sampling process and Phenograph clustering up to 5 times to achieve robustness in the results. Through our strategy, we effectively analyzed up to 2500 cells per sample. Despite the level of stringency imposed by the repeated sampling and clustering process, we observed little dispersion in the results indicating that our findings were robust (CV of 5% for cells with an antibody-secreting phenotype and 12% for cells with a memory B phenotype). We did not observe significant differences in the average and SD of the signal for each marker nor in the frequencies of the major immune compartments across repeated analyses.

Statistical Analyses
Statistical significance was performed using GraphPad Prism or R. Statistical tests used are reported in the figure legends. Comparisons between DSAPOS, DSA-negative recipients (DSANEG), and DSAPOSAMR-positive recipients (AMRPOS) groups at the same time point were performed by unpaired t tests or ANOVA (nonparametric tests were also performed yielding similar results). Differences are considered significant at P < 0.05.

RESULTS
Recipients
All included subjects were unsensitized, first-time pediatric recipients of kidney transplants who received the same induction and similar immunosuppression therapy (Table 1).

Adherence to CNI maintenance immunosuppression, as determined by cyclosporine or tacrolimus IPV at 6 months posttransplant and onward, did not differ in DSAPOS versus DSANEG recipients or in DSAPOS patients who developed (DSAPOSAMRPOS) or not (DSAPOSAMR-negative recipients (AMRNEG)) AMR (Figure S1, SDC, http://links.lww.com/TXD/A217, see Adherence to CNI Therapy in the Materials and Methods section). The majority of patients received deceased donor renal transplants (Table 1). Ten recipients developed DSA posttransplant, and 11 remained DSA negative over the 5-year follow-up period. Baseline characteristics did not differ significantly between DSAPOS and DSANEG recipients (Table 1). Recipients who developed DSA had increased rates of acute cellular rejection, AMR, and graft loss during the 4-year follow-up period (Table 2).

TABLE 1
Baseline characteristics of donors and recipients

|                     | Total (n = 21) | DSAPOS (n = 10) | DSANEG (n = 11) | P   |
|---------------------|---------------|-----------------|-----------------|-----|
| **Donors**          |               |                 |                 |     |
| Age, y              | 15.0 ± 12.0   | 11.0 ± 5.8      | 18.5 ± 15.1     | 0.15|
| Male, n (%)         | 14 (67)       | 8 (80)          | 6 (54)          | 0.36|
| Living, n (%)       | 3 (33)        | 0 (0)           | 3 (27)          | 0.21|
| **Recipients**      |               |                 |                 |     |
| Age, y              | 10.9 ± 5.2    | 10.8 ± 6.4      | 10.9 ± 4.3      | 0.96|
| Male, n (%)         | 13 (62)       | 8 (80)          | 5 (46)          | 0.18|
| Race (%)            |               |                 |                 | 1.00|
| White               | 19 (90)       | 9 (90)          | 10 (91)         |     |
| Black               | 1 (5)         | 0 (0)           | 1 (9)           |     |
| Hispanic            | 1 (5)         | 1 (10)          | 0 (0)           |     |
| Primary renal disease, n (%) |     |                 |                 | 0.22|
| Alport syndrome     | 1 (5)         | 1 (10)          | 0 (0)           |     |
| ANCA+ vasculitis    | 2 (10)        | 0 (0)           | 2 (18)          |     |
| ARPKD               | 2 (10)        | 2 (20)          | 0 (0)           |     |
| CAKUT               | 10 (48)       | 6 (60)          | 4 (36)          |     |
| FSGS                | 2 (10)        | 1 (10)          | 1 (9)           |     |
| Interstitial nephropathy | 1 (5) | 0 (0)           | 1 (9)           |     |
| Nephrophosphatis     | 3 (14)        | 1 (10)          | 2 (18)          |     |
| Mo on dialysis      | 17.3 ± 11.1   | 17.2 ± 10.2     | 17.5 ± 12.4     | 0.96|
| Cold ischemia time, min | 791.3 ± 309.9 | 816.8 ± 184.9  | 768.2 ± 400.0  | 0.72|
| HLA mismatch (A + B + DQ + DR) | 4.5 ± 1.3 | 4.6 ± 1.3 | 4.4 ± 1.4 | 0.68|

All donors were white. No patient had a peak panel-reactive antibody >20%. Continuous variables are represented as mean ± SD. Categorical variables are expressed as a percentage. ANCA, antineutrophil cytoplasmic antibodies; ARPKD, autosomal recessive polycystic kidney disease; CAKUT, congenital anomalies of the kidney and urinary tract; DSA, donor-specific antibody; DSANEG, DSA-negative recipients; DSAPOS, DSA-positive recipients; FSGS, focal segmental glomerulosclerosis; HLA, human leukocyte antigen; SD, standard deviation.

DSA Antibody Levels and C1q and C3d Binding
Baseline characteristics (Table S2, SDC, http://links.lww.com/TXD/A217) of MFI levels at the time of DSA detection (9.5 ± 3.4 mo posttransplant) were similar between recipients who did and did not develop AMR (Table S3, SDC, http://links.lww.com/TXD/A217; 13 686.6 ± 9300.8 vs 11 375.4 ± 4234.5, respectively; P = 0.63). All DSAPOS patients developed antibodies reactive against HLA class II antigens. Three recipients also had DSA against HLA class I, but those antibodies were developed either at the time of DSA detection (n = 1) or after the onset of class II DSA (n = 2; Table S3, SDC, http://links.lww.com/TXD/A217). DSA levels remained generally stable over time, except in 3 DSAPOSAMRPOS patients who showed an increase in their levels.

The majority of DSAs were targeted against HLA-DQ and bound C1q (Table 3). Three of the 5 DSAPOSAMRPOS recipients
had DSA that bound C3d upon initial DSA detection, while only 1 DSAPOS AMRNEG recipient had C3d-binding DSA (Table 3).

**Frequencies of Major Immune Compartments**

We analyzed the evolution of 9 major immune compartments in peripheral blood (Figure 2) using an unbiased algorithm (Phenograph49; see Materials and Methods section). The resultant high-dimensional clustered data were visualized in 2 dimensions with preserved single-cell resolution using viSNE (Figure 2A). Once all clusters were defined as belonging to a major immune compartment, the cellular frequencies were calculated for each patient at all time points.

Overall, cell frequencies of major immune compartments remained relatively stable throughout the 3 time points and were similar to the cell frequencies previously reported by our group in adult kidney transplant recipients during the first year after transplantation.46 Major immune subsets were not significantly different between DSAPOS and DSANEG recipients except for monocytes, which were elevated in DSAPOS recipients before DSA detection (Figure 2). While no single cluster reciprocally increased in the DSANEG group, an increase in several lymphocyte populations in DSANEG recipients collectively accounted for the elevated monocytes in DSAPOS recipients before DSA development (Figure S2, SDC, http://links.lww.com/TXD/A217). B cells declined over time in both groups, but only DSANEG subjects had a statistically significant decrease in total B cells from 2 months posttransplant to the time of DSA detection.

**DSAPOS and DSANEG Recipients Have Similar Frequencies of Circulating CD4+ and CD8+ T-Cell Subsets**

Although we did not detect any significant differences between CD4+ or CD8+ T cells in DSAPOS versus DSANEG recipients (Figure 2), we performed a second level of unbiased clustering within those compartments using 20 additional markers to determine whether any subpopulations differed between the 2 recipient groups (Table S1, SDC, http://links.lww.com/TXD/A217; Figure 3A and B). The cells from all recipients were pooled together, and 8 CD4+ (Figure 3A and B) and 7 CD8+ (Figure 4A and B) T-cell subpopulations were defined based on relative expression of the clustering markers. The subclusters were then demultiplexed to extract the frequency of each subpopulation for each patient over time (Figures 3B and 4B). Similar to our findings for the major immune compartments, no significant differences in CD4+ and CD8+ T-cell subpopulations were detected.

Of note, CD4+ T cells with a T-follicular helper-like (T FH) phenotype (see Figure 3, clusters 2, 11, and 19 for markers) were not increased in the peripheral circulation of DSAPOS recipients despite their importance in antibody production.50,51 The percentages of regulatory T cells (T REG) also did not significantly differ between DSA POS and DSANEG recipients at the analyzed time points. When we stratified DSAPOS recipients according to the future development of AMR, we could not

**Table 2. Allograft outcomes of patients by DSA status**

|                      | Total (n = 21) | DSAPOS (n=10) | DSANEG (n = 11) | P     |
|----------------------|---------------|---------------|-----------------|-------|
| eGFR posttransplant  |               |               |                 |       |
| 3 mo                 | 102.7 ± 19.6  | 98.8 ± 24.9   | 106.2 ± 13.6    | 0.42  |
| 6 mo                 | 101.9 ± 20.5  | 97.7 ±24.9    | 105.8 ± 15.8    | 0.39  |
| 9 mo                 | 98.2 ± 18.6   | 94.7 ± 22.7   | 101.3 ± 14.3    | 0.45  |
| 12 mo                | 98.2 ± 18.8   | 95.2 ± 23.2   | 100.8 ± 14.3    | 0.52  |
| DGF, n (%)           | 2 (10)        | 1 (10)        | 1 (9)           | 1.00  |
| ACR, n (%)           | 3 (33)        | 3 (30)        | 0 (0)           | 0.09  |
| ACR time posttransplant, mo | 5 (24) | 17.0 ± 1.0 | N/A | 0.01* |
| AMR, n (%)           | 5 (50)        | 5 (50)        | 0 (0)           |       |
| AMR time posttransplant, mo | 27.4 ± 8.0 | N/A | N/A |       |
| Graft loss, n (%)    | 3 (33)        | 3 (30)        | 0 (0)           | 0.09  |
| Graft loss time posttransplant, mo | 48.0 ± 25.1 | N/A | N/A |       |

*P < 0.05 by unpaired t test for continuous variables or Fisher exact test for categorical variables.

eGFR was estimated via the Schwartz formula.48 Categorical variables are expressed as a percentage. Continuous variables are represented as mean ± SD.

**Table 3. DSA specificities for DSAPOS recipients**

|                      | Total (n = 10) | AMRPOS (n = 5) | AMRNEG (n = 5) | P     |
|----------------------|---------------|---------------|---------------|-------|
| HLA specificity      |               | DQ5 DQ6      | DQ41 0501     |       |
|                      |               | DQA1 0505*   | DQ7           |       |
|                      |               | DQ8a         | DQ7           |       |
|                      |               | DQ9          | DQ5           |       |
|                      |               | A2 B58 B37 DR10* | DQ2       |       |
| C1q binding, n (%)   | 9 (90)        | 5 (100)      | 4 (80)        | 1.00  |
| C3d binding, n (%)   | 4 (40)        | 3 (60)       | 1 (20)        | 0.52  |

*Recipients who lost their grafts.

Recipients not listed in any particular order. Variables are expressed as a percentage.

AMR, antibody-mediated rejection; AMRNEG, AMR negative patients; AMRPOS, AMR positive patients; DSA, donor-specific antibody; DSAPOS, DSA-positive recipients; eGFR, estimated glomerular filtration rate; N/A, non-applicable; SD, standard deviation.
detect any significant differences in any of the CD4+ or CD8+ T-cell compartments (not shown).

**B Cells With Antibody-Secreting and Memory Phenotypes Are Increased in DSAPOS Recipients Before AMR Development**

Within the B-cell compartment, we identified 10 distinct B-cell subpopulations, including B cells with a naive (IgD+CD27−),52 regulatory (CD25hi),53,54 antibody-secreting (IgD−CD27+CD38+),55 and memory (IgD−CD27+CD38−)52 phenotype. We did not detect statistically significant differences at any time point in B-cell subpopulations between DSAPOS and DSANEG recipients (Figure 5B).

However, we noticed a trend toward increased B cells with antibody-secreting or memory phenotypes at the time of DSA in DSAPOS recipients compared with DSANEG individuals. We then hypothesized that stratification of DSAPOS recipients according to the development of AMR within the first 5 years posttransplant would elucidate cells potentially responsible for this immunological process. B cells with antibody-secreting or memory phenotypes were significantly increased at the time of DSA detection only in recipients who developed AMR >1 year later. Of note, these cells were present in the peripheral blood ≈18 months before the onset of AMR diagnosis by biopsy (Table 2). None of the major immune compartments differed significantly at any time points between DSAPOS patients who did or did not develop AMR. DSA levels at 2 and 3 years after transplant in DSAPOS patients who developed AMR did not significantly differ compared with those of DSAPOS patients who did not develop AMR (DSA at 2 y: 12 170 ± 7722 vs 8540 ± 7799, *P = 0.48; at 3 y: 11 970 ± 3997 vs 11 600 ± 7214 MFI; *P = 0.92, respectively). The 3 patients who lost their grafts developed AMR and had high levels of B cells with antibody-secreting and memory phenotypes.

**DISCUSSION**

Identifying cellular immune mediators responsible for AMR in kidney transplant recipients has proven difficult due to the limited number of markers that can be probed using flow cytometry. We utilized unbiased CyTOF analyses to comprehensively characterize the immune phenotype in pediatric kidney transplant recipients using serial samples collected before and at the time of DSA development. While CyTOF has been used with great success in the oncologic sciences, its implementation in human solid organ transplantation has been limited to a few studies.46,56 Herein, we discovered that, while percentages of circulating B cells with an antibody-secreting or memory phenotype did not significantly differ between DSAPOS and DSANEG recipients before DSA detection, they were enriched at the time of DSA detection only in recipients who developed AMR >1 year later.

![Figure 2: Frequencies of major immune compartments. A. Representative viSNE plots of peripheral blood mononuclear cells (PBMC) colored by their relative expression of cell markers used to define major immune clusters (populations defined on the right). B. Percentages of major immune compartments based on the summation of Phenograph clusters (see Materials and Methods, n = 21 subjects). Error bars represent SEM. Comparisons between DSAPOS and DSANEG (*P < 0.05, **P < 0.01 by unpaired t test) and with baseline (▲P < 0.05 by paired t test). DC, Dendritic Cells; DSA, donor-specific antibody; DSANEG, DSA-negative recipients; DSAPOS, DSA-positive recipients; NK, Natural Killer; pDC, Plasmacytoid Dendritic Cells; SEM, standard error of the mean.](https://www.transplantationdirect.com/issue)
It is well established that DSA positivity portends inferior allograft outcomes due to the increased risk of AMR. However, the factors leading to dnDSA development and progression to AMR are unclear. Half of the DSAPOS recipients in our cohort developed AMR despite similar baseline characteristics, immunosuppression regimens, and donor/recipient HLA matches (Table 1). Nonadherence has been implied as a major risk factor for the development of DSA, especially in pediatric recipients. IPV in cyclosporine and tacrolimus levels was within target ranges and similar across all recipients (Figure S1, SDC, http://links.lww.com/TXD/A217), suggesting that nonadherence was not a major determinant for dnDSA development in our cohort.

DSA levels at the time of DSA detection were similar as well, making this information of little value to risk-stratify DSA recipients who will develop AMR (Table S3, SDC, http://links.lww.com/TXD/A217). Others have reported that the ability to fix and activate the complement cascade, as determined by C1q and C3d binding, identifies pathogenic DSA and correlates with shorter allograft survival. However, 9 out of the 10 recipients in our cohort had DSA that bound C1q (Table 3). More DSAPOSAMRPOS recipients had C3d-binding DSA, but this trend did not reach statistical significance (P = 0.52). Therefore, in this pediatric population, C1q and C3d staining did not prove to be useful predictors of AMR development.

B cells with memory or antibody-secreting phenotype were the only cell subsets that we identified as differentially increased in DSAPOS recipients who later developed AMR. These cells were identified based on their absence of IgD and expression of CD27—signifying mature B cells—and differential expression of CD38. CD38 is a highly conserved type II glycoprotein that possesses pleiotropic effects on B-cell function and maturation. Antibody-secreting cells, including plasma cells and immature plasmablasts, express high levels of CD38, whereas memory B cells, a long-lasting B-cell subset capable of differentiating into antibody-secreting B cells and producing high-affinity antibodies...
upon antigen re-encounter, lack CD38 expression. Our data confirm and expand to a pediatric population previous evidence by Luque et al showing increased donor-reactive memory B cells in the circulation of adult DSAPOS recipients with ongoing AMR. Intriguingly, these authors identified donor-reactive memory B cells by Elispot, while they failed to identify a difference in any of the B cell subsets measured by flow cytometry. The increased sensitivity of the CyTOF technique when compared with flow cytometry provides the opportunity to identify and quantify low-frequency lymphocyte populations in peripheral blood. Indeed, we were able to probe for differences in 9 major immune compartments and 25 lymphocyte subpopulations using CyTOF.

Our comprehensive immune phenotypic characterization by CyTOF also allowed us to test for any relationship between circulating T-cell subsets and DSA development. Unexpectedly, we did not detect differences in any of the CD4+ or CD8+ T-cell subsets between DSAPOS and DSANEg recipients at any time point, including circulating T FH—a specialized T-cell subset integral to efficient antibody production that assists B-cell differentiation into plasma cells—and T REG. Different groups have attempted to find a relationship between circulating T FH or T REG and graft outcomes, but results, invariably generated using flow cytometry, have been inconsistent so far. Our comprehensive, unbiased approach on serially collected samples does not support such a relationship, at least in pediatric patients.

Our study has some limitations. The sample size was relatively small, and we did not validate our results in an independent cohort. However, we resampled and reanalyzed the B-cell clusters 5 times to ensure that our findings were statistically robust. CyTOF allowed us to conduct comprehensive immune phenotyping with increased sensitivity for low-expressed markers. Although perfectly suited to extract the most information from a reduced number of samples, it cannot overcome the limitations associated with a small number of patients. We also have not determined that the B cells with a memory or antibody-secreting phenotype are specific to the HLA antigens that the DSAs were against. Lack of functional data on B cells with a memory or antibody-secreting...
phenotype represents another limitation, although the cell surface markers used to classify these cells are well established in the literature.67,68 Our phenotypic data complement functional studies by others showing that memory B cells can be detected in the circulation before DSA development.52 Future studies will be required to define antigen specificity of B cells with memory or antibody-secreting phenotype.

Overall, the present study demonstrates that circulating B cells with an antibody-secreting and memory phenotype increase in DSAPOS recipients before the development of AMR. We also show that these cells are detectable in the peripheral circulation at the time of DSA onset, more than a year before biopsy-proven rejection, highlighting a possible role for aggressive B-cell–targeted immunosuppression in these at-risk recipients. These pilot results warrant further validation in larger cohort studies.

REFERENCES

1. Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group. KDIGO clinical practice guideline for the care of kidney transplant recipients. Am J Transplant. 2009;9(Suppl 3):S1–S155.
2. Kasiske BL, Zeier MG, Chapman JR, et al; Kidney Disease: Improving Global Outcomes. KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary. Kidney Int. 2010;77:299–311.

3. Lamb KE, Lodhi S, Mei-er-Kriessche HU. Long-term renal allograft survival in the United States: a critical reappraisal. Am J Transplant. 2011;11:450–462.

4. Lodhi SA, Lamb KE, Mei-er-Kriessche HU. Solid organ allograft survival improvement in the United States: the long-term does not mirror the dramatic short-term success. Am J Transplant. 2011;11:1226–1235.

5. Mei-er-Kriessche HU, Schold JD, Srinivas TR, et al. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant. 2004;4:378–383.

6. Van Arendonk KJ, Boyarsky BJ, Orandi BJ, et al. National trends over 25 years in pediatric kidney transplant outcomes. Pediatrics. 2014;134:604–609.

7. Zhang R. Donor-specific antibodies in kidney transplant recipients. Clin J Am Soc Nephrol. 2018;13:182–192.

8. Wiene C, Gibson IW, Blydt-Hansen TD, et al. Evolution and clinical pathological correlates of de novo donor-specific HLA antibody post kidney transplantation. Am J Transplant. 2012;12:1157–1167.

9. Hart A, Smith JM, Skeans MA, et al. Kidney. Am J Transplant. 2016;16(Suppl 2);11–46.

10. Hart A, Smith JM, Skeans MA, et al. OPUTR/SRTTR 2016 Annual Data Report: kidney. Am J Transplant. 2018;18(Suppl 1);18–113.

11. Chehade H, Pascual M. The challenge of acute antibody-mediated rejection in kidney transplantation. Transplantation. 2016;100:264–265.

12. Montgomery RA, Loupy A, Segev DL. Antibody-mediated rejection: new approaches in prevention and management. Am J Transplant. 2018;18(Suppl 3);3–17.

13. Gaston RS, Cecka JM, Kasiske BL, et al. Evidence for antibody-mediated injury as a major determinant of late kidney allograft failure. Transplantation. 2010;90:68–74.

14. Nankivel BJ, Kuppers DR. Diagnosis and prevention of chronic kidney allograft loss. Lancet. 2011;378:1428–1437.

15. Sautenet B, Blancho G, Büchler M, et al. Evidence for chronic kidney allograft loss. Kidney Int. 2011;77:299–311.

16. Valenzuela NM, Reed EF. Antibodies in transplantation: the effects of HLA and Non-HLA antibody binding and mechanisms of injury. In: Zachary AA, Leffell MS, editors. Transplantation Immunology: Methods and Protocols. Totowa, NJ: Humana Press; 2013:41–70.

17. Kumbala D, Zhang R. Essential concept of transplant immunology for clinical practice. World J Transplant. 2013;3:113–118.

18. Thomas KA, Valenzuela NM, Reed EF. The perfect storm: HLA antibodies, complement, fcyrs, and endothelium in transplant rejection. Trends Mol Med. 2015;21:319–329.

19. Comoli P, Cioni M, Tagliamacco A, et al. Acquisition of C3d-binding activity by de novo donor-specific HLA antibodies correlates with graft loss in nonsensitized pediatric kidney recipients. Am J Transplant. 2016;16:2106–2116.

20. Chen G, Sequeira F, Tyas MB. Novel C1Q assay reveals a clinically relevant subset of human leukocyte antigen antibodies independent of immunoglobulin G strength on single antigen beads. Hum Immunol. 2011;72:849–858.

21. Yabu JM, Higgins JP, Chen G, et al. C1q-fixing human leukocyte antigen antibodies are specific for predicting transplant glomerulopathy and late graft failure after kidney transplantation. Transplantation. 2011;91:342–347.

22. Freitas MO, Ribudello LM, Ozawa M, et al. The role of immunoglobulin-G subclasses and C1Q in de novo HLA-DQ donor-specific antibody kidney transplantation outcomes. Transplantation. 2013;95:1113–1119.

23. Sutherland SM, Chen G, Sequeira FA, et al. Complement-fixing donor-specific antibodies identified by a novel C1Q assay are associated with allograft loss. Pediatr Transplant. 2012;16:12–17.

24. Sicard A, Ducreu S, Rabeyrin M, et al. Detection of C3d-binding donor-specific anti-HLA antibodies at diagnosis of humoral rejection predicts renal graft loss. J Am Soc Nephrol. 2015;26:457–467.

25. Loupy A, Lefaucheur C, Vernerey D, et al. Complement-binding anti-HLA antibodies and kidney-allograft survival. Eur J Med. 2013;369:1215–1226.

26. Lee H, Han E, Choi AR, et al. Clinical impact of complement (C1q, C3d) binding de novo donor-specific anti-HLA antibody in kidney transplant recipients. PLOS One. 2018;13:e0207434.

27. Okabe Y, Noguchi H, Miyamoto K, et al. Preformed C1q-binding donor-specific anti-HLA antibodies and graft function after kidney transplantation. Transplant Proc. 2011;43:3460–3464.

28. Moreno Gonzales MA, Minneberg DG, Smith BH, et al. Comparison between total IgG, C1q, and C3d single antigen bead assays in detecting Class I complement-binding anti-HLA antibodies. Transplant Proc. 2017;49:2031–2035.

29. Barnouil J, Rodenburg A, Staeck O, et al. Clinical outcome of patients with de novo C1q-binding donor-specific HLA antibodies after renal transplantation. Transplantation. 2017;101:2165–2174.

30. Guidicelli G, Guerville F, Lepreux S, et al. Non-complement-binding de novo donor-specific anti-HLA antibodies and kidney allograft survival. J Am Soc Nephrol. 2016;27:615–625.

31. Buismans AM, de Rond CG, Oztürk K, et al. Long-term presence of memory B-cells specific for different vaccine components. Vaccine. 2009;28:179–186.

32. Crotty S, Aubert RD, Gildeewell J, et al. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. J Immunol Methods. 2006;284:111–122.

33. Tarlinton D, Good-Jacobson K. Diversity among memory B cells: origin, consequences, and utility. Science. 2013;341:1205–1211.

34. McHeyzer-Williams MG, Ahmed R. B cell memory and the long-lived plasma cell. Curr Opin Immunol. 1999;11:1172–1179.

35. Chong AS, Sciammas R. Memory B cells in transplantation. Transplantation. 2013;96:68–74.

36. Lúcia M, Luque S, Crespo E, et al. Preformed circulating HLA-specific memory B cells predict high risk of humoral rejection in kidney transplantation. Kidney Int. 2015;88:874–887.

37. Haas M, Sis B, Racusen LC, et al. Banff Meeting Report Writing Committee. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. Am J Transplant. 2014;14:272–283.

38. McHeyzer-Williams MG, Ahmed R. B cell memory and the long-lived plasma cell. Curr Opin Immunol. 1999;11:1172–1179.

39. Chong AS, Sciammas R. Memory B cells in transplantation. Transplantation. 2013;96:68–74.

40. Visentin J, Vignata M, Daburon S, et al. Deciphering complement interference in anti-human leukocyte antigen antibody detection with flow beads assays. Transplantation. 2014;98:625–631.

41. Shuker N, van Gelder T, Hesselink DA. Intra-patient variability in tacrolimus trough concentrations and renal function decline in pediatric renal transplant recipients. Pediatr Transplant. 2012;16:613–618.

42. Lavin Y, Kobayashi S, Leader A, et al. Inmate immune landscape in early lung adenocarcinoma by paired single-cell analyses. Cell. 2017;169:750.e17–765.e17.

43. Fribourg F, Fischman C, Anderson L, et al. pal stimulators of de novo donor-specific antibodies in nonsensitized pediatric recipients receiving a first kidney transplant. Transpl Int. 2014;27:667–673.

44. Prytula AA, Bouts AH, Mathot RA, et al. Intra-patient variability in tacrolimus trough concentrations and renal function decline in pediatric renal transplant recipients. Pediatr Transplant. 2012;16:613–618.

45. Tarlinton D, Good-Jacobson K. Diversity among memory B cells: origin, consequences, and utility. Science. 2013;341:1205–1211.

46. McHeyzer-Williams MG, Ahmed R. B cell memory and the long-lived plasma cell. Curr Opin Immunol. 1999;11:1172–1179.

47. Chong AS, Sciammas R. Memory B cells in transplantation. Transplantation. 2013;96:68–74.

48. Schwartz GJ, Haycock GB, Edelmann CM Jr, et al. A simple estimate of glomerular filtration rate in children derived from body length and plasma creatinine. Pediatrics. 1976;58:259–263.

49. Levine JH, Simonds EF, Bendall SC, et al. Data-driven phenotypic dissections of AML reveals progenitor-like cells that correlate with prognosis. Cell. 2015;162:184–197.

50. Campbell DJ, Kim CH, Butcher EC. Separable effector T cell populations specialized for B cell help or tissue inflammation. Nat Immunol. 2001;2:876–881.
51. Qi H. T follicular helper cells in space-time. *Nat Rev Immunol.* 2016;16:612–625.

52. Luque S, Lúcia M, Melilli E, et al. Value of monitoring circulating donor-reactive memory B cells to characterize antibody-mediated rejection after kidney transplantation. *Am J Transplant.* 2019;19:365–380.

53. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity.* 2015;42:607–612.

54. van de Veen W, Stanic B, Yaman G, et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol.* 2013;131:1204–1212.

55. Matsumoto M, Baba A, Yokota T, et al. Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. *Immunity.* 2014;41:1040–1051.

56. Yabu JM, Siebert JC, Maecker HT. Immune profiles to predict response to desensitization therapy in highly HLA-sensitized kidney transplant candidates. *PLoS One.* 2016;11(4):e0153355.

57. Worthington JE, Martin S, Al-Husseini DM, et al. Posttransplantation production of donor HLA-specific antibodies as a predictor of renal transplant outcome. *Transplantation.* 2003;75:1034–1040.

58. Lefaucheur C, Loupy A, Hill GS, et al. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. *J Am Soc Nephrol.* 2010;21:1398–1406.

59. Bartel G, Wahrmann M, Schwaiger E, et al. Solid phase detection of C4d-fixing HLA antibodies to predict rejection in high immunological risk kidney transplant recipients. *Transpl Int.* 2013;26:121–130.

60. Lawrence C, Willcombe M, Brookes PA, et al. Preformed complement-activating low-level donor-specific antibody predicts early antibody-mediated rejection in renal allografts. *Transplantation.* 2013;95:341–346.

61. Jacquot S. CD27/CD70 interactions regulate T dependent B cell differentiation. *Immunol Res.* 2000;21:23–30.

62. Funaro A, Spagnoli GC, Ausiello CM, et al. Involvement of the multilineage CD38 molecule in a unique pathway of cell activation and proliferation. *J Immunol.* 1990;145:2390–2396.

63. Zupo S, Rugari E, Dono M, et al. CD38 signaling by agonistic monoclonal antibody prevents apoptosis of human germinal center B cells. *Eur J Immunol.* 1994;24:1218–1222.

64. Deaglio S, Vasisti T, Bergui L, et al. CD38 and CD100 lead a network of surface receptors relaying positive signals for B-CLL growth and survival. *Blood.* 2005;105:3042–3050.

65. Deaglio S, Capobianco A, Bergui L, et al. CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. *Blood.* 2003;102:2146–2155.

66. Nutt SL, Hodgkin PD, Tarlinton DM, et al. The generation of antibody-secreting plasma cells. *Nat Rev Immunol.* 2015;15:160–171.

67. Jelinek DF, Siplawski JB, Lipsky PE. Human peripheral blood B lymphocyte subpopulations: functional and phenotypic analysis of surface IgD positive and negative subsets. *J Immunol.* 1986;136:83–92.

68. Liu YJ, Barthélemy C, de Bouteiller O, et al. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity.* 1995;2:239–248.

69. Cano-Romero FL, Laguna Goya R, Utrero-Rico A, et al. Longitudinal profile of circulating T follicular helper lymphocytes parallels anti-HLA sensitization in renal transplant recipients. *Am J Transplant.* 2012;12:3989–97.

70. de Graa GN, Dieterich M, Hesselink DA, et al. Follicular T helper cells and humoral reactivity in kidney transplant patients. *Clin Exp Immunol.* 2015;180:329–340.

71. Chenouard A, Chesneau M, Bui Nguyen L, et al. Renal operational tolerance is associated with a defect of blood Tfh cells that exhibit impaired B cell help. *Am J Transplant.* 2017;17:1490–1501.

72. Alberu J, Vargas-Rojas MI, Morales-Buenrostro LE, et al. De novo donor-specific HLA antibody development and peripheral CD4(+) CD25(high) cells in kidney transplant recipients: a place for interaction? *J Transplant.* 2012;2012:302539.