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Comprehensive Phenotyping and Cytokine Production of Circulating B Cells Associate Resting Memory B Cells With Early Antibody-mediated Rejection in Kidney Transplant Recipients

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Background. B cells play a crucial role in kidney transplantation through antibody production and cytokine secretion. To better understand their impact on kidney transplantation, this retrospective study aimed to characterize circulating B-cell phenotypes and cytokine production in a cohort of kidney transplant patients to identify whether pretransplant donor-specific antibodies (DSAs) or biopsy-proven rejection is associated with different B-cell profiles. **Methods.** Pretransplant cryopreserved peripheral blood mononuclear cells were obtained from 96 kidney transplant recipients, of whom 42 had pretransplant DSAs. The cells underwent surface marker staining using a 33-color spectral flow cytometry panel for B-cell phenotyping. Simultaneously, cells were stimulated for interleukin-10, tumor necrosis factor-α, and interleukin-6 production, and analyzed with a 6-color panel. **Results.** Rejection was linked to decreased naive B cells and increased plasmablasts, $CD27^+$ memory B cells, and memory B-cell subsets (all $P < 0.04$) compared with no rejection. Cytokine-producing B cells and immune regulatory molecule expression showed no significant differences. Multivariate analysis identified resting memory B cells ($CD27^+CD21^+$) and pretransplant DSAs as significantly associated with rejection ($P = 0.01$; odds ratio [OR], 1.07; $P = 0.02$; OR, 3.10, respectively). Cox regression analysis revealed resting memory B cells were associated with early antibody-mediated rejection ($P = 0.04$; OR, 1.05). **Conclusions.** B-cell subset distributions differed between patients with and without rejection. Resting memory B-cell frequency was associated with increased early antibody-mediated rejection risk, whereas cytokine production and immune checkpoint expression did not influence rejection. The results suggest that B-cell subset composition could aid in rejection risk assessment and serve as a potential pretransplant diagnostic parameter.

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The authors declare no conflicts of interest.

D.A. designed and performed the research, analyzed the data, and wrote the article. J.C.v.d.B. participated in the data acquisition, reanalyzed the biopsy data, and reviewed the article. T.B. performed the experiment and reviewed the article. C.G.B. designed the experiment and reviewed the article. N.I. participated in data analysis and reviewed the article. R.L. participated in data analysis, article writing, and reviewing. P.H., S.B., and J.-S.S. participated in the study design, data analysis, and article writing and reviewing.

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In conclusion, our study highlights the important role of B cells in kidney transplantation, with a particular focus on the potential of resting memory B cells as a predictive marker for early AMR. The results suggest that resting memory B cells may serve as indicators of rejection risk, though this potential requires validation. Future studies should adopt a longitudinal approach, tracking the shifts in B-cell populations and functions over time to better understand their dynamic roles in transplant outcomes. Ultimately, this study underscores the integral role of B cells in kidney transplant immunology while emphasizing the need for more comprehensive research to translate this knowledge into clinical practices for predicting and preventing rejection.

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The central role of B cells in antibody-mediated rejection (AMR) has sparked increased interest in researching their complex functions in transplant immunology,[1,2] with growing attention on the clinical impact of B-cell subsets, namely memory B cells ($CD27^{+}$),[3] transitional B cells ($CD38^{hi}$),[4] and IL-10-producing regulatory B cells (Bregs).[5-11]

B cells have been extensively studied as potential predictors of transplant rejection risk, with numerous studies exploring their role in graft outcomes.[4,12-16] Although some research work suggests that specific B-cell subsets, particularly donor-specific memory B cells, may indicate rejection likelihood and graft survival,[17] the clinical utility of this approach remains uncertain. Our previous study, for instance, failed to confirm associations reported in earlier research, highlighting the complexity of this field.[18] However, the balance between effector B cells (eg, memory B cells) and Bregs appears crucial for graft outcomes. For example, increased frequencies of transitional and memory Breg subsets ($CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$) have been linked to prolonged graft survival and reduced rejection risk,[4,15] potentially contributing to long-term graft stability or operational tolerance.[1,7,19] Aside from the aforementioned subsets and their role in transplant immunology, B cells express various immune checkpoints that regulate their activation, differentiation, and survival.[1] These include costimulatory molecules such as CD40 and CD86, which interact with T cells to promote activation, and receptors such as B-cell activating factor receptor, transmembrane activator and CAML interactor, and interleukin (IL)-21R, which are crucial for B-cell survival and differentiation.[1,2,20-23] Conversely, coinhibitory molecules like programmed cell death protein 1 and B- and T-lymphocyte attenuator can suppress B-cell activation when engaged.[24,25] The balance and interplay between these various checkpoints could influence B-cell responses in transplantation, affecting both alloimmunity and tolerance.

To explore the complex roles of B cells in kidney transplant immunology, we used a dual flow cytometry approach on pretransplant peripheral blood mononuclear cells (PBMCs) from kidney transplant recipients (KTRs). A 33-color B cell–specific spectral flow cytometry panel was used to analyze the B-cell subset phenotypes and immune checkpoint expression, whereas a conventional panel was used to assess cytokine production by B cells (IL-10, IL-6, tumor necrosis factor [TNF-α]) after in vitro stimulation. This comprehensive method aimed to identify potential rejection risk predictors by characterizing both phenotypic and functional aspects of B cells in KTRs with and without rejection and donor-specific antibodies (DSAs).

MATERIALS AND METHODS

Patient Selection

This retrospective study used patient data and materials originally included during the PROCARE 1 multicenter study conducted from 1995 to 2005.[26] Our subsequent experiments and analysis focused specifically on KTRs who underwent transplantation at the University Medical Center Groningen (UMCG) during the PROCARE 1 study period. KTRs were divided into 2 groups based on their pretransplant DSA status, with positive DSAs indicating high risk and negative DSAs indicating standard risk. The study relied on previously collected materials within the PROECARE consortium and the TransplantLines biobank, adhering to the protocol approved by the UMCG medical ethical committee (METc 2014/077) and the Declaration of Helsinki principles.

A total of 42 patients with pretransplant DSAs and 54 patients without them were identified for this study. The patients were matched at the group level based on age, sex, and induction therapy. The presence of DSAs was assessed using the Luminex single antigen bead assay on serum samples. All transplants were conducted after negative T- and B-cell complement-dependent cytotoxicity crossmatch tests. Additionally, PBMC collection was performed using the standard techniques at the transplant immunology laboratory of the UMCG, and HLA typing was performed for both patients and their respective donors as part of the main consortium study.[26,27] An overview of the baseline patient characteristics is provided in Table 1.

Biological materials, including PBMCs and serum samples, were generally collected 6–36 h before transplantation. In certain instances where these samples were not available, the most recent serum samples before transplantation were used instead for DSA detection. Further details regarding patient inclusion and the experimental design are provided in Figure 1.

Biopsy and Rejection Assessment

In this study, indication biopsies from 58 KTRs were reassessed using the 2019 BANFF scoring criteria to align with current standards for defining rejection.[28] In cases where multiple biopsies were available for a single patient, the initial biopsy that was previously evaluated as having or suspected of having rejection based on the standards at the time was reevaluated. The median time between transplantation and biopsy was 19 d, with an interquartile range of 10 to 97 d.

Flow Cytometry

As shown in Figure 1, the protocol was divided into 2 parts: subset phenotyping using spectral flow cytometry and cytokine production using classical flow cytometry. The following section provides a detailed description of both components.

Spectral Panel and Staining

A 35-color B-cell spectral flow cytometry panel, previously described by Bonasia et al,[29] was adapted for this study to phenotype B-cell subsets and map the expression of surface immune regulatory molecules. The panel was slightly adjusted to fit the objective of this study, resulting in a 33-color panel instead of 35. Cryopreserved PBMC staining was performed using a slightly modified method to that outlined by Bonasia et al[29] to account for the new changes, with antibody concentrations determined through prior titration experiments. **Table S1** (**SDC,** http://links.lww.com/TXD/A745) provides a detailed summary of the concentrations and staining sequence. After the staining procedure, the cell phenotype was analyzed using a Cytek Aurora full spectrum flow cytometer (Cytek Biosciences, CA), and 1 million events were examined per sample. A healthy control sample was included in each experiment to account for batch variations.

Manual Gating Analysis of Spectral Data

Spectral flow cytometry data were analyzed using manual gating in the OMIQ (Dotmatics, Boston, MA) web program (www.omiq.ai; 2024 interface). The identification of $CD19^{+}CD20^{+/-}$ B cells and the subsequent subsets followed previously published classification strategies[3,30] as well as

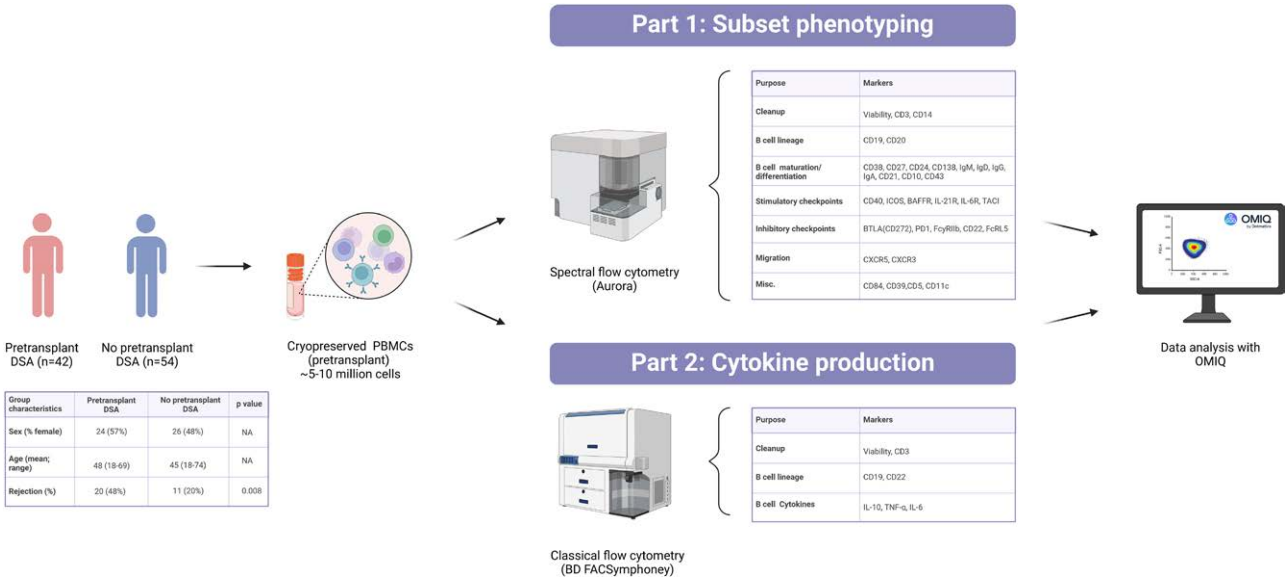
TABLE 1.

Patient and transplant baseline characteristics

Characteristics	Study population (n = 96)	Rejection (n = 31)	No rejection (n = 65)
Age, y	44.8 ± 13.8	42.6 ± 12.5	44.9 ± 13.9
Female sex	50 (52%)	16 (52%)	34 (52%)
Kidney disease			
Glomerulonephritis	27 (28%)	8 (26%)	19 (29%)
Cystic and congenital disease	16 (17%)	4 (13%)	12 (18%)
Vascular kidney disease	18 (19%)	7 (23%)	11 (17%)
Pyelonephritis and interstitial nephritis	13 (13%)	4 (13%)	9 (14%)
Diabetic kidney disease	5 (5%)	1 (3%)	4 (6%)
Other	17 (18%)	7 (23%)	10 (15%)
Dialysis			
Hemodialysis	52 (54%)	19 (61%)	33 (51%)
Peritoneal dialysis	41 (43%)	12 (39%)	29 (45%)
None	3 (3%)	0 (0%)	3 (5%)
Dialysis time, y	4.1 ± 3.1	3.9 ± 3.1	4.0 ± 3.0
Type of donor			
Deceased	87 (91%)	29 (94%)	58 (89%)
Living	9 (9%)	2 (6%)	7 (11%)
Donor age, y	40.8 ± 15.5	41.7 ± 15.5	40.8 ± 15.5
Cold ischemia time, h	19.5 ± 8.6	19.5 ± 8.4	19.5 ± 8.7
Sensitizing events			
Pregnancy only	31 (32%)	8 (26%)	23 (35%)
Retransplant only	28 (29%)	9 (29%)	19 (29%)
Pregnancy and retransplant	4 (4%)	2 (6%)	2 (3%)
Not sensitized or not known (unreported pregnancy, blood transfusion, etc)	33 (34%)	12 (35%)	21 (32%)
PRA			
Pretransplant PRA, %	9.4 ± 19.9	8.6 ± 18.4	9.5 ± 19.9
Highest PRA, %	23.8 ± 31.8	22.6 ± 31.0	23.9 ± 31.9
Induction therapy			
Antithymocyte globulin	36 (38%)	8 (26%)	28 (43%)
Basiliximab	37 (39%)	10 (32%)	27 (42%)
Other	1 (1%)	1 (3%)	0 (%)
No induction	22 (23%)	12 (39%)	10 (15%)
Maintenance immunosuppression type			
Steroids	96 (100%)	31 (100%)	65 (100%)
Tacrolimus	19 (20%)	6 (19%)	13 (20%)
Cyclosporine	76 (79%)	25 (81%)	51 (78%)
Mycophenolate mofetil	87 (91%)	27 (87%)	60 (92%)
Other	3 (3%)	2 (6%)	1 (1%)
Immunological characteristics			
Total HLA mismatches (HLA-A, -B, -DR; max 6)	2.6 ± 1.4	2.6 ± 1.4	2.6 ± 1.42
Class I (max 4)	1.8 ± 1.1	1.7 ± 1.1	1.8 ± 1.14
Class II (max 2)	0.8 ± 0.6	0.81 ± 0.6	0.8 ± 0.61
Pretransplant DSA	42 (44%)	20 (65%)	22 (34%)
Immunodominant DSA type	DQB1*04:02, DQA1*04:01	DQB1*04:02, DQA1*04:01	B*27:05
Immunodominant DSA mean fluorescence intensity	18700	18700	12800

Values are presented as n (%) or mean ± SD.
DSA, donor-specific antibody; PRA, panel-reactive antibody.

the one detailed by Bonasia et al,[29] with a few modifications when necessary. **Figure S1** (**SDC,** http://links.lww.com/TXD/A745) provides a visual representation of the gating strategy. The expression of immune regulatory markers in B cells and subsets was determined through manual gating of the median fluorescence intensity (MFI) using 2 distinct approaches. For markers expressed by most or all B cells, such as B cell activating factor receptor, FcRγIIB (CD32), CD40, B- and T-lymphocyte attenuator, and CD22, gates for positive MFI were established using the $CD19^{+}CD20^{+/-}$ B-cell subset, with almost all cells within this gate included in the analysis. In contrast, for markers expressed by only a fraction of B cells, including transmembrane activator and CAML interactor, Fc receptor-like protein 5, IL-6R, programmed cell death protein 1, ICOSL, IL-21R, and CD86, a negative subset ($CD19^{-}CD20^{-}$) was selected as a "blank" to define the MFI positivity threshold. This threshold was then applied to assess positive expression on the $CD19^{+}CD20^{+/-}$ B cells and subsets. **Figure S2** (**SDC,** http://links.lww.com/TXD/A745) provides an example of this gating process for both approaches.



Group characteristics	Pretransplant DSA	No pretransplant DSA	p value
Sex (% female)	24 (57%)	26 (48%)	NA
Age (mean; range)	48 (18-69)	45 (18-74)	NA
Rejection (%)	20 (48%)	11 (20%)	0.008

FIGURE 1. An overview of the experimental design, including patient selection and the technical approaches used in this study. Created with BioRender.com. BAFF-R, B cell activating factor receptor; BTLA, B- and T-lymphocyte attenuator; DSA, donor-specific antibody; FcRL5, Fc receptor-like protein 5; FcRγIIB, Fc gamma receptor IIB; ICOSL, inducible T-cell costimulator; IL-6, interleukin 6; IL-10, interleukin 10; IL-21R, interleukin 21 receptor; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; TACI, transmembrane activator and CAML interactor; TNF-α, tumor necrosis factor alpha.

Cytokine Production and Staining

In the second part of the protocol, PBMCs were stimulated in vitro to produce cytokines following a previously published method.[31] Subsequent to the stimulation, the cells were stained for viability using Zombie UV Fixable Viability dye (BioLegend), followed by surface marker staining with antibodies against human CD3 (UCHT1; BioLegend), CD19 (HIB19, eBioscience), and CD22 (HIB22, BD Bioscience). The cells were then fixed and permeabilized using the FIX & PERM Cell Permeabilization Kit (Invitrogen, ThermoFisher Scientific) and stained for intracellular cytokines with fluorochrome-conjugated antibodies against IL-10 (JES3-9D7, BioLegend), TNF-α (Mab11, BioLegend), and IL-6 (MQ2-13A5, eBioscience). Intracellular cytokine production was analyzed using a BD FACSymphony flow cytometer (BD Biosciences, NJ), with 100 000 events examined per sample. The brefeldin A-only samples were used as controls for gate setting.

Manual Gating Analysis of Cytokine Data

The data generated from the cytokine production experiments were analyzed using manual gating in OMIQ web program. Initially, the B-cell subset ($CD19^{+}CD22^{+/-}$) was gated from the live $CD3^{-}$ cell population, then, the cytokine-producing B cells within the main B-cell group were further classified into distinct categories based on their cytokine production profiles. **Figure S3** (**SDC**, http://links.lww.com/TXD/A745) provides a visual representation of the gating strategy.

Statistical Analysis

Statistical analyses and data visualization were performed using SPSS (IBM Corp, NY) version 28 software, GraphPad Prism (Dotmatics, Boston, MA) version 9.0 software, and RStudio (R language version 4.3.2 with RStudio build 764; Posit Software, Boston, MA). To compare the frequencies of the B-cell subsets and cytokine-producing B cells, as well as the MFI of the immune checkpoints, a Mann-Whitney *U* test was used to identify statistical significance between groups. The Benjamini and Hochberg false discovery rate (FDR) correction test with a *Q* value of 5% was used to account for multiple hypothesis testing, with a *P* value of <0.05 considered to be statistically significant for all tests.

To assess whether the B-cell subsets were independently associated with rejection, univariate and multivariate logistic regression models were generated. In the univariate model, predictors (ie, immunological or clinical baseline risk factors) were individually associated with rejection using the Wald test. Risk factors with a *P* value of <0.1 were then included in the multivariate model. The final multivariate model was established through a stepwise backward elimination until each predictor showed an association with rejection with a *P* value of <0.05.

To further explore the relationship between resting memory B cells in rejection patients, we used a multistep analytical approach. First, we used the Kruskal-Wallis test with Benjamini and Hochberg FDR correction to compare patients with and without pretransplant DSAs. Next, to determine which type of rejection had a stronger correlation with resting memory B cells, we conducted Mann-Whitney *U* tests (with FDR correction). These tests compared AMR (including mixed cases) and T cell–mediated rejection (TCMR) patients against the no-rejection group. We also assessed the influence of transplantation eras on the association between resting memory B cells and rejection using the same method. Finally, to evaluate the impact of AMR timing and resting memory B-cell percentages, we performed a Cox regression analysis.

A heatmap using the Ward clustering method was generated with the "tidyverse" R package to visualize the immune regulatory checkpoints on B-cell subsets. The MFI data were aggregated by computing log-transformed fold changes (logFC) between different groups (eg, patients with pretransplant DSA and those without). The resulting data were scaled and visualized as a heatmap. Importantly, for an immune regulatory checkpoint to be considered on a

particular subset, at least 10 cells per sample needed to be positive for the regulatory molecule to ensure reliability of the reported data.

RESULTS

Biopsy Profiles and Patient and Graft Outcome Measures

In this study, a total of 96 KTRs were included. Among these, 58 patients underwent indication biopsies that were assessed for rejection. The patient and transplant outcomes, including allograft pathology data such as the Banff rejection diagnosis, are depicted in Table 2. The study included cases of AMR (n = 16) and TCMR (n = 15). Among the 16 patients with AMR, 6 patients had mixed AMR/TCMR.

TABLE 2.

Graft outcomes including biopsy-proven rejection, the overall patient survival, and death censored graft loss up to 10 y posttransplant

Outcomes	Study population (n = 96)
No. of indication biopsies assessed	58 (60%)
Rejection (all)	31 (32%)
Total TCMR (including double cases)	21 (22%)
IA	3 (3%)
IB	6 (7%)
IIA	4 (4%)
IIB	3 (3%)
Borderline	5 (5%)
Total AMR (including double cases)	16 (17%)
Acute	10 (10%)
Chronic active	6 (7%)
Overall patient survival	66 (69%)
Graft survival	70 (73%)
Death censored graft loss	26 (27%)

AMR, antibody-mediated rejection; TCMR, T cell–mediated rejection.

Analysis of the Frequencies of the Circulating B-cell Subsets and Their Association With Pretransplant DSAs and Rejection

In the first part of the analysis, we investigated whether the presence of pretransplant DSAs affected the frequency of circulating B cells (Figure 2). Our findings indicated a trend toward decreased naive B-cell frequencies and increased memory B-cell compartments, including memory Bregs, resting memory, and unswitched memory in the pretransplant DSA group. However, these trends did not reach statistical significance after corrections ($P = 0.14$ for all). In the second part of our analysis, we explored whether variations in B-cell subset frequencies influenced transplant outcomes, namely biopsy-proven rejection (Figure 3). In the rejection group and after corrections, we observed a statistically significant decrease in the frequency of naive B cells ($P = 0.039$) and an increased frequency of plasmablasts ($P = 0.039$). Additionally, the memory B-cell compartment showed elevated frequencies in the general CD27⁺ memory B cells ($P = 0.039$), memory Bregs ($P = 0.037$), resting memory ($P = 0.037$), and activated memory ($P = 0.037$) subsets. There was also a trend toward a higher frequency of unswitched memory (IgM^+IgD^+) and IgA-producing memory B cells in the rejection group; however, this did not reach statistical significance after correction ($P = 0.057$ for both).

No Differences in the Frequencies of Cytokine-producing B Cells

To determine whether the circulating B cells possess functional properties, we examined their cytokine production capacity alongside the immunophenotyping process. This was achieved using cells from the spectrally phenotyped samples, as depicted in Figure 1. We first evaluated whether the presence of pretransplant DSAs was linked to the cytokine profile of circulating B cells after stimulation (Figure 4). Next, we assessed whether specific frequencies of cytokine-producing B cells influenced the occurrence of rejection (Figure 5). In both

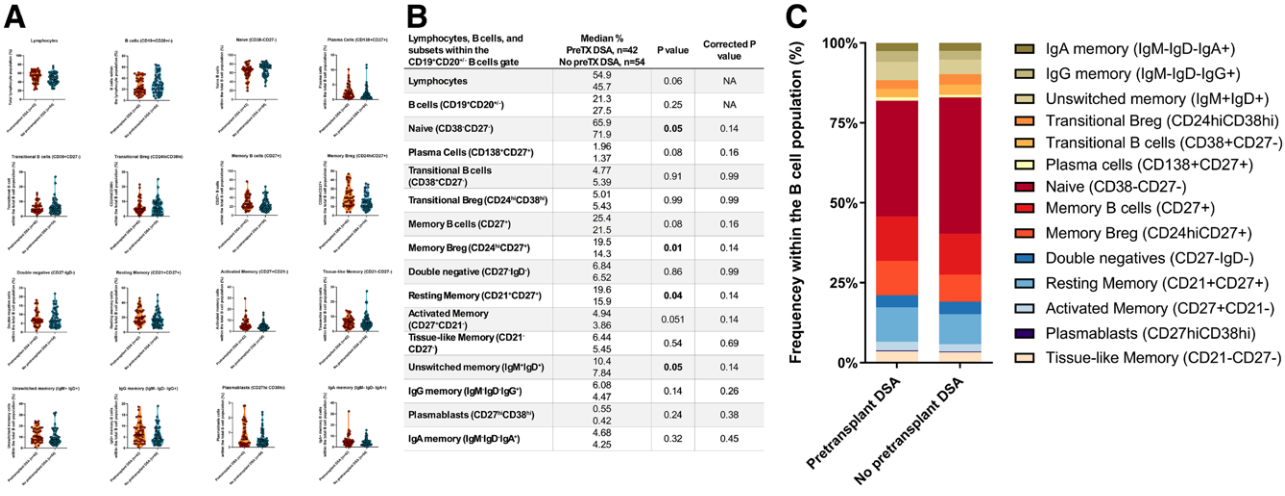
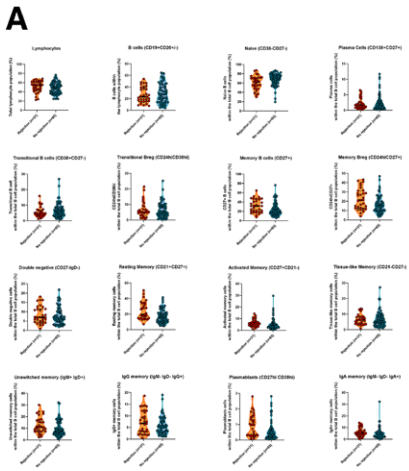


FIGURE 2. Comparison of lymphocytes and B-cell subset frequencies identified through manual gating analysis in patient groups with and without pretransplant DSAs. A, Violin plots illustrating the frequency differences of lymphocytes, $CD19^+CD20^{+/-}$ B cells, and major B-cell subsets between KTRs with and without preTx DSAs, as identified through manual gating analysis. The dark line within each violin plot represents the median. B, The statistical results from the Mann-Whitney test and subsequent corrections. C, A frequency plot depicting the distribution of major B-cell subsets within the total B-cell population of each group after normalization. Breg, regulatory B cell; DSA, donor-specific antibody; KTR, kidney transplant recipient; preTx, pretransplant.



B

Lymphocytes, B cells, and subsets within the CD19+CD20+/− B cells gate	Median % Rejection, n=31 / No rejection, n=65	P value	Corrected P value
Lymphocytes	54.8 48.9	0.19	NA
B cells (CD19+CD20+/−)	23.3 27.3	0.64	NA
Naive (CD38+CD27+)	62.7 71.0	**0.01**	**0.039**
Plasma Cells (CD138+CD27+)	1.67 1.73	0.94	0.95
Transitional B cells (CD38+CD27−)	4.53 5.59	0.25	0.34
Transitional Breg (CD24hiCD38hi)	5.10 5.13	0.81	0.88
Memory B cells (CD27+)	31.5 20.1	**0.01**	**0.039**
Memory Breg (CD24hiCD27+)	21.1 14.5	**0.01**	**0.037**
Double negative (CD27−IgD−)	6.94 6.55	0.52	0.67
Resting Memory (CD21+CD27+)	19.4 15.3	**0.003**	**0.037**
Activated Memory (CD27+CD21−)	5.76 3.68	**0.008**	**0.037**
Tissue-like Memory (CD21−CD27−)	6.26 5.33	0.64	0.76
Unswitched memory (IgM+IgD+)	10.9 7.58	**0.03**	0.057
IgG memory (IgM−IgD−IgG+)	6.70 5.01	0.15	0.22
Plasmablasts (CD27hiCD38hi)	0.95 0.40	**0.01**	**0.039**
IgA memory (IgM−IgD−IgA+)	5.14 4.08	**0.03**	0.057

C

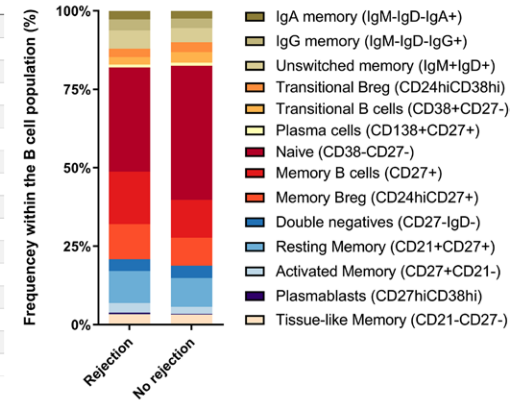
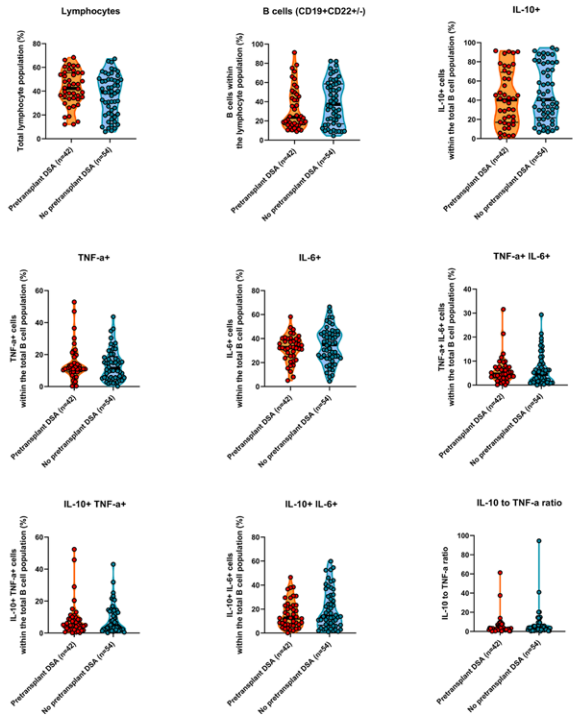


FIGURE 3. Comparison of lymphocytes and Bcell subset frequencies identified through manual gating analysis in patient groups with and without rejection. A, Violin plots illustrating the frequency differences of lymphocytes, CD19+CD20+/− B cells, and major B-cell subsets between KTRs with and without rejection, as identified through manual gating analysis. The dark line within each violin plot represents the median. B, The statistical results from the Mann-Whitney test and subsequent corrections. C, A frequency plot depicting the distribution of major B-cell subsets within the total B-cell population of each group after normalization. Breg, regulatory B cell; DSA, donor-specific antibody; KTR, kidney transplant recipient.

A



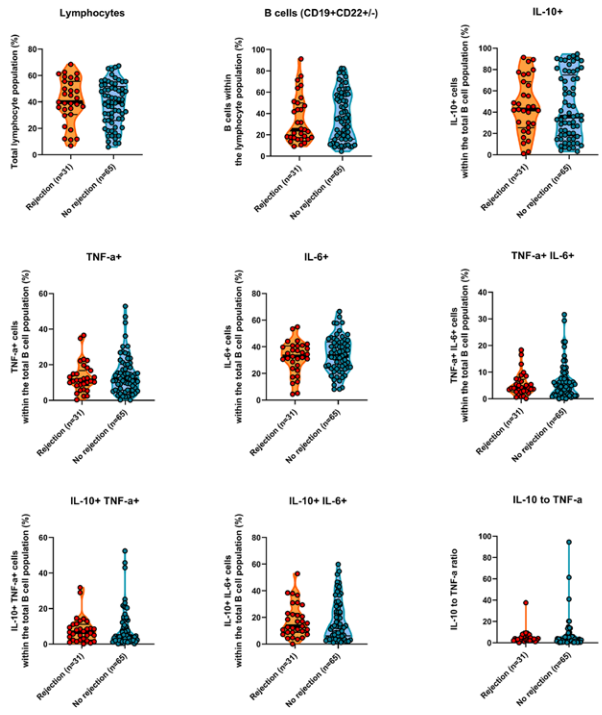
B

Lymphocytes, B cells, and cytokine-producing subsets within the CD19+CD22+/− B cells gate	Median % PreTX DSA, n=42 / No preTX DSA, n=54	P value	Corrected P value
Lymphocytes	42.3 39.0	0.11	NA
B cells (CD19+CD22+/−)	23.9 37.3	0.26	NA
IL-10+	40.4 40.6	0.39	0.76
TNF-a+	11.3 11.4	0.63	0.76
IL-6+	32.5 34.9	0.49	0.76
TNF-a+IL-6+	4.54 4.55	0.62	0.76
IL-10+TNF-a+	4.96 4.70	0.94	0.95
IL-10+IL-6+	12.5 13.8	0.37	0.76
IL-10 to TNF-a ratio	2.75 3.90	0.07	NA

FIGURE 4. Comparison of cytokine-producing lymphocytes and B-cell subsets identified through manual gating analysis in patient groups with and without pretransplant DSAs. A, Violin plots depicting the frequency differences of lymphocytes, CD19+CD22+/− B cells, and cytokine-producing B cells, including the IL-10 to TNF-α ratio, between KTRs with and without preTx DSAs, as determined through manual gating analysis. The dark line within each violin plot represents the median. B, The statistical results from the Mann-Whitney test and subsequent corrections are presented. DSA, donor-specific antibody; IL-10, interleukin 10; KTR, kidney transplant recipient; preTx, pretransplant; TNF-α, tumor necrosis factor alpha.

analyses, we found no significant differences in the frequencies of B cells producing IL-6, IL-10, and TNF-α after stimulation. The ratio of anti- to proinflammatory cytokines (and vice versa) has been studied before in transplantation as another measure of the immune function of B cells.[15] Therefore, we explored the ratio of IL-10 to TNF-α in this study as well but did not observe differences between KTRs in either the pretransplant DSA analysis (Figure 4) or the rejection analysis (Figure 5). Although no statistically significant differences were observed, the analysis confirmed that these B cells possess functional capabilities, as they produced both pro- and anti-inflammatory cytokines on stimulation.

A



B

Lymphocytes, B cells, and cytokine-producing subsets within the CD19⁺CD22⁺/⁻ B cells gate	Median % Rejection, n=31 No rejection, n=65	P value	Corrected P value
Lymphocytes	43.8 39.5	0.47	NA
B cells (CD19⁺CD22⁺/⁻)	24.9 35.4	0.56	NA
IL-10⁺	42.6 35.9	0.68	0.85
TNF-a⁺	11.3 11.9	0.76	0.85
IL-6+	32.7 33.1	0.59	0.85
TNF-a⁺IL-6⁺	4.03 5.16	0.68	0.85
IL-10⁺TNF-a⁺	6.66 4.51	0.63	0.85
IL-10⁺IL-6⁺	13.4 13.2	0.84	0.85
IL-10 to TNF-a ratio	3.18 3.46	0.33	NA

FIGURE 5. Comparison of cytokine-producing lymphocytes and B-cell subsets identified through manual gating analysis in patient groups with and without rejection. A, Violin plots depicting the frequency differences of lymphocytes, CD19⁺CD22⁺/⁻ B cells, and cytokine-producing B cells, including the IL-10 to TNF-α ratio, between KTRs with and without rejection, as determined through manual gating analysis. The dark line within each violin plot represents the median. B, The statistical results from the Mann-Whitney test and subsequent corrections. IL-10, interleukin 10; KTR, kidney transplant recipient; TNF-α, tumor necrosis factor alpha.

No Differences in the Expression of Immune Regulatory Molecules on Circulating B Cells and Subsets

We aimed to determine whether the circulating B cells in the included KTRs exhibited variations in their immune regulatory molecules. Our analysis began with a comparison of the expression of these molecules within the CD19⁺CD20⁺/⁻ B cells among KTRs with and without DSAs, and those with and without rejection. We found no significant differences in MFI values between these groups, as illustrated in Figures 6 and 7. Next, we extended our investigation to examine the expression of these checkpoints within each B-cell subset. Our analysis revealed no statistically significant differences in the expression of these immune regulatory molecules within the B-cell subsets. To provide a clearer visualization of checkpoint expressions within the subsets, we constructed heatmaps presenting the logFC in expression. These heatmaps are displayed in **Figure S4** (**SDC,** http://links.lww.com/TXD/A745) for the pretransplant DSA analysis and **Figure S5** (**SDC,** http://links.lww.com/TXD/A745) for the rejection analysis.

Resting Memory B-cell Frequencies are Associated With Rejection

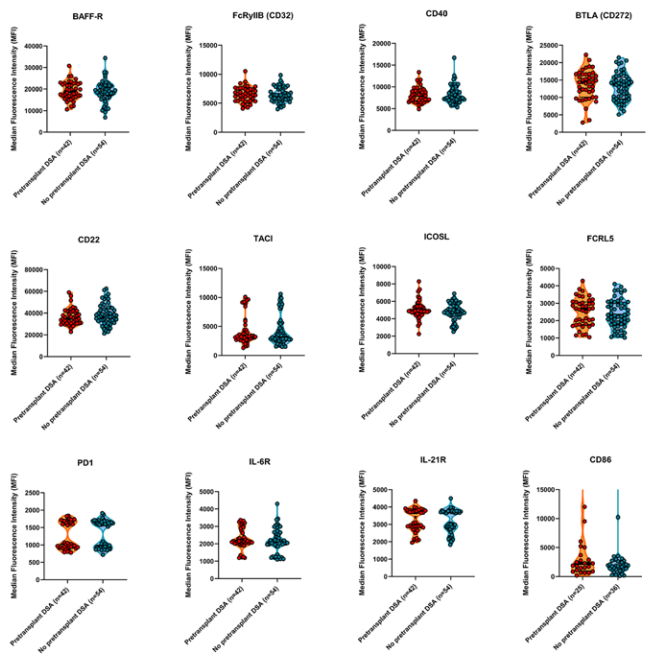
The results in Figure 3 indicate that the distribution of B-cell subsets could have a more pronounced influence on the occurrence of rejection. To further explore whether the impact of the frequency of the B-cell subsets on rejection was independent of other factors, we conducted univariate and multivariate logistic regression analyses. A detailed overview of the results of the univariate and multivariate analyses are presented in Table 3. The final model revealed that, in addition to pretransplant DSA status ($P = 0.02$; odds ratio [OR], 3.10), the frequencies of resting memory B cells (CD27⁺CD21⁺) were significantly associated with rejection ($P = 0.01$; OR, 1.07). The result of this analysis was interesting because it indicated that each additional percentage of resting memory B cells was associated with 7% higher risk of developing rejection, suggesting a potentially harmful effect of these memory B cells on the transplant outcomes.

Resting Memory B Cells: Further Insights Into Their Relationship With Rejection

Since the multivariate model revealed an interesting link between resting memory B cells and rejection, we conducted further analyses to explore this relationship for a more nuanced understanding.

First, we determined if the association between resting memory B cells and rejection differed in older versus newer samples from KTRs. Most of our samples (76%; n = 73) were from 2000 to 2005, with the remainder from 1995 to 1999. To examine potential differences, we divided the samples into 2 groups: an older era (1995–1999, n = 23) and a newer era (2000–2005, n = 73). Using the Mann-Whitney test, we analyzed the influence of resting memory B-cell frequencies on rejection in both groups. The older rejection group (n = 8) showed a median of 33.95%, whereas the newer rejection group (n = 23) had a median of 17.70%, both with a P value of 0.032 after FDR correction. These findings suggest that the association between resting memory B cells and rejection remains consistent across differing transplantation eras.

A

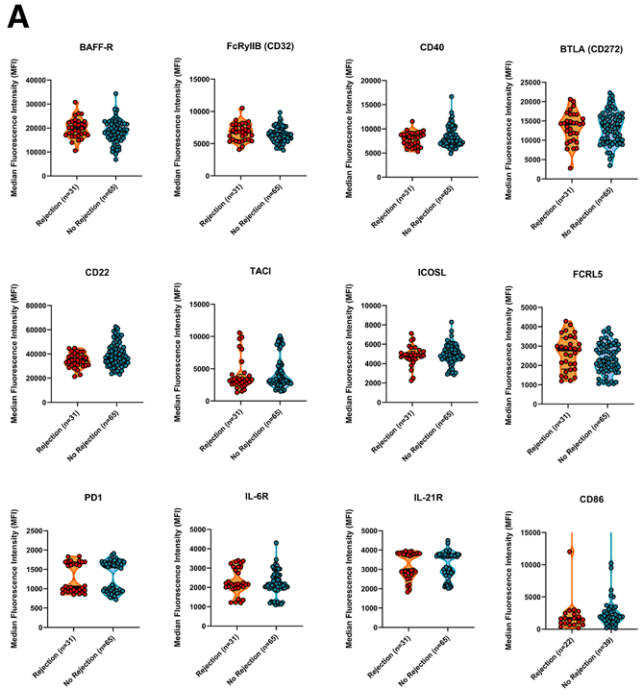


B

Regulatory checkpoints within the CD19⁺CD20⁺/⁻ B cells gate	Median FI PreTX DSA No preTX DSA	P value	Corrected P value
BAFF-R	18883 18668	0.70	0.88
FcRyIIB (CD32)	6439 6367	0.75	0.88
CD40	8059 7620	0.88	0.89
BTLA	14312 13554	0.32	0.88
CD22	35215 37014	0.27	0.88
TACI	3317 3138	0.62	0.88
ICOSL	4904 4833	0.34	0.88
FcRL5	2655 2312	0.58	0.88
PD-1	1043 1578	0.80	0.88
IL-6R	2162 2124	0.46	0.88
IL-21R	3391 3038	0.42	0.88
CD86	2578 2228	0.19	0.88

FIGURE 6. Comparison of immne regulatory checkpoints within the CD19⁺CD20⁺ B cells identified through manual gating analysis in patient groups with and without pretransplant DSAs. A, Violin plots displaying the differing expression of regulatory molecules on CD19⁺CD20⁺/⁻ B cells between groups with and without preTx DSAs. The dark line within each violin plot represents the median. B, Accompanying these plots is a table presenting the relevant statistical findings from the Mann-Whitney test and subsequent corrections. BAFF-R, B cell activating factor receptor;BTLA, B- and T-lymphocyte attenuator; DSA, donor-specific antibody; FcRL5, Fc receptor-like protein 5; FcRγIIB, Fc gamma receptor IIB; ICOSL, inducible T-cell costimulator; IL-6, interleukin 6; IL-21R, interleukin 21 receptor; preTx, pretransplant; PD-1, programmed cell death protein 1; TACI, transmembrane activator and CAML interactor.

A



B

Regulatory checkpoints within the CD19⁺CD20⁺/⁻ B cells gate	Median FI Rejection No rejection	P value	Corrected P value
BAFF-R	19863 18471	0.27	0.79
FcRyIIB (CD32)	6797 6376	0.18	0.73
CD40	8197 7520	0.65	0.79
BTLA	14274 13605	0.78	0.86
CD22	34728 36956	0.06	0.67
TACI	3314 3231	0.57	0.79
ICOSL	4861 4880	0.64	0.79
FcRL5	2819 2299	0.11	0.67
PD-1	1039 1577	0.96	0.97
IL-6R	2165 2112	0.35	0.79
IL-21R	2969 3120	0.53	0.79
CD86	2050 2609	0.44	0.79

FIGURE 7. Comparison of immne regulatory checkpoints within the CD19⁺CD20⁺ B cells identified through manual gating analysis in patient groups with and without rejection. A, Violin plots displaying the differing expression of regulatory molecules on CD19⁺CD20⁺/⁻ B cells between groups with and without rejection. The dark line within each violin plot represents the median. B, Accompanying these plots is a table presenting the relevant statistical findings from the Mann-Whitney test and subsequent corrections. BAFF-R, B cell activating factor receptor; BTLA, B- and T-lymphocyte attenuator; DSA, donor-specific antibody; FcRL5, Fc receptor-like protein 5; FcRγIIB, Fc gamma receptor IIB; ICOSL, inducible T-cell costimulator; IL-6, interleukin 6; IL-21R, interleukin 21 receptor; preTx, pretransplant; PD-1, programmed cell death protein 1; TACI, transmembrane activator and CAML interactor.

TABLE 3.

Univariate and multivariate analyses using basic logistic regression models of the rejection predictors, namely baseline characteristics and B-cell subsets

	Univariate analysis		Multivariate analysis	
Variable	**Odds ratio (95% CI)**	***P***	**Odds ratio (95% CI)**	***P***
Age (1-y increase)	0.98 (0.95-1.01)	0.33		
Sex				
Female	1	–		
Male	1.03 (0.44-2.42)	0.95		
Induction therapy				
No	1	–		
Yes	0.23 (0.11-0.78)	**0.014**		
Donor age (1-y increase)	0.99 (0.97-1.03)	0.85		
Donation type				
Live	1	–		
Deceased	1.75 (0.34-8.96)	0.50		
Prior kidney transplant				
No	1	–		
Yes	1.15 (0.47-2.84)	0.76		
Pregnancy				
No	1	–		
Yes	0.60 (0.17-2.13)	0.43		
Cold ischemia time (1-h increase)	1.01 (0.96-1.06)	0.67		
No. of HLA mismatches	1.08 (0.79-1.46)	0.62		
Pretransplant DSA				
No	1	–	1	–
Yes	3.55 (1.45-8.72)	**0.006**	3.10 (1.21-7.95)	**0.02**
Time to biopsy (1-y increase)	1.08 (0.93-1.26)	0.30		
B-cell subsets (1% increase)				
Naive	0.96 (0.93-0.99)	**0.02**		
Memory	1.04 (1.00-1.07)	**0.03**		
Memory Bregs	1.05 (1.01-1.09)	**0.01**		
Resting memory	1.08 (1.03-1.13)	**0.002**	1.07 (1.02-1.12)	**0.01**
Plasmablasts	2.17 (1.12-4.21)	**0.02**		
Activate memory	1.05 (0.96-1.16)	0.27		

The bolded values indicate statistical significance.
Breg, regulatory B cell; CI, confidence interval; DSA, donor-specific antibody.

Interestingly, the older group exhibited a higher percentage of pretransplant resting memory B cells, despite having fewer cases of AMR. This unexpected result could be due to various factors, such as changes in PBMC isolation protocols or modifications to induction therapy regimens over time.

We then assessed whether the association with rejection was independent of DSAs. Our findings indicated that resting memory B cells were significantly elevated in both rejection groups compared with the no-rejection group, with the rejection with DSAs (n = 20) showing a median of 23.8% ($P = 0.05$) and the rejection without DSAs (n = 11) showing a median of 17.7% ($P = 0.05$). Notably, there was no significant difference in resting memory B-cell levels between the 2 rejection groups ($P = 0.81$), which corroborated our findings from Figure 2.

Next, we examined the specific type of rejection associated with these cells. The AMR group (n = 16) exhibited a statistically significant increase in resting memory B-cell frequencies compared with the no-rejection group (median 27% versus 15.3%, $P = 0.03$), whereas the TCMR group (n = 15) showed elevated levels that were not statistically significant (median 17.7% versus 15.3%, $P = 0.08$). This suggested that the elevated resting memory B-cell levels observed in the total rejection group (n = 31) were primarily driven by the AMR cases.

Finally, we investigated the connection between resting memory B-cell frequency and rejection timing using a Cox regression analysis. In this study, most rejection cases (27/31) occurred within the first 2 y posttransplant. Only a few patients (n = 4) experienced rejection later: 3 developed AMR and 1 developed TCMR at more distant time points. Because our previous analysis revealed that resting memory B cells were closely associated with AMR rather than TCMR, our Cox regression focused only on AMR cases. We first assessed the relation between timing of AMR and resting memory B cells across all cases (n = 16) as depicted in (Figure 8) and found no clear statistical association ($P = 0.15$; OR, 1.03). However, when we excluded cases occurring >5 y posttransplant (n = 3), a stronger association emerged ($P = 0.04$; OR, 1.05), indicating that higher frequencies of resting memory B cells were significantly linked to early AMR (Figure 9). These findings suggest that resting memory B cells could serve as potential indicators for early rejection risk, particularly in cases of early AMR.

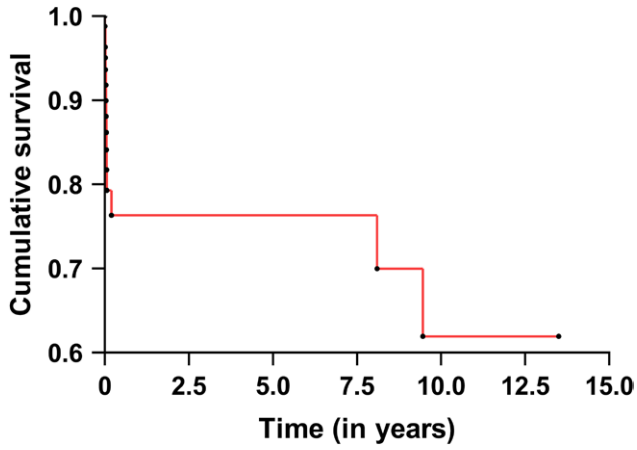


FIGURE 8. Cox regression analysis linking AMR timing with resting memory B-cell frequencies during 15 y, showing a nonsignificant trend ($P = 0.15$) and OR of 1.03. AMR, antibody-mediated rejection; OR, odds ratio.

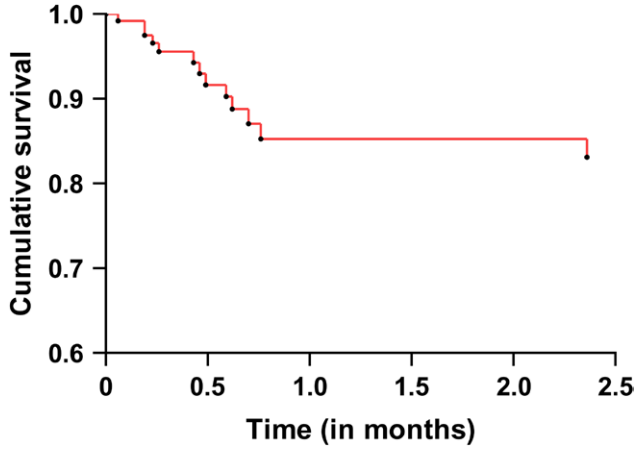


FIGURE 9. Cox regression analysis linking AMR timing with resting memory B-cell frequencies during 18 mo, revealing a significant association ($P = 0.04$) and OR of 1.05. AMR, antibody-mediated rejection; OR, odds ratio.

DISCUSSION

The analysis of B-cell subset composition in peripheral blood offers valuable insights into an individual's immune status and helps illustrate the role of immune cells in transplant outcomes. Our study used this potential by using a comprehensive 33-color spectral flow cytometry panel and a functional in vitro stimulation assay to investigate the distribution of B-cell subsets, their regulatory molecules expression, and associated cytokine production in KTRs. We specifically examined the relationship of these circulating B cells with pretransplant DSAs and posttransplant rejection. By combining these advanced techniques, our study contributes to a more nuanced understanding of B cells in transplantation, paving the way for improved risk assessment approaches.

The analysis of the spectral flow cytometry data revealed variations in B-cell subset frequencies among groups with and without rejection. Our results revealed differences in frequencies of memory B cells, naive B cells, and plasmablasts, with the most notable differences observed in the resting memory B cells. Evidence from published studies suggests that memory B-cell frequencies tend to increase in patients who develop rejection, particularly AMR. Irure-Ventura et al[16] reported significantly higher pretransplant levels of switched memory B cells in patients who developed AMR compared with no-rejection patients. They also observed a reduced ratio of naive to memory B cells in AMR patients compared with no-rejection patients and healthy controls, suggesting decreased frequencies of naive B cells in KTRs with AMR.[16] Louis et al[3] demonstrated that activated memory B cells ($CD27^{+}CD21^{-}$) coexpressing T-bet were associated with AMR due to their differentiation into antibody-producing plasma cells. Although our study did not replicate these specific findings for the active and the switched (represented by IgG^{+} and IgA^{+}) memory B cells, we observed an increase in other memory B-cell subsets in patients with biopsy-proven rejection. Of particular interest were the resting memory B cells, which our analysis identified as significantly and independently associated with rejection (specifically with early AMR), alongside pretransplant DSA status. Resting memory B cells (defined as $CD45^{+}CD19^{+}CD21^{+}CD27^{+}$)[32] are long-persisting, highly proliferative cells capable of rapid expansion and antibody production upon activation.[32,33] Their role in transplantation is not well understood, but they likely contribute to DSA production, key mediators of rejection.[32,34] This finding is interesting for future risk assessment techniques in kidney transplantation, given that the current practice primarily focuses on serum HLA antibody detection, potentially neglecting the cellular aspect.

We also assessed the potential variations in the frequency of transitional B cells ($CD38^{hi}$) between the rejection and no-rejection groups and found no statistical associations, which was in line with previous findings in other studies that assessed this in pretransplant PBMCs of KTRs.[4,35,36] However, because our study did not include posttransplant samples, we cannot ascertain whether a posttransplant association exists (namely at the 3 mo posttransplant) as reported in the literature, wherein a decrease in the transitional B-cell population was linked to biopsy-proven rejection.[4,35-37]

Finally, we examined the relationship between pretransplant DSA and B-cell subset distribution. Although not statistically significant, we observed trends suggesting an increase in memory B-cell subsets ($CD27^{+}$ memory, unswitched memory, memory Breg, and resting memory) and a decrease in naive B cells in patients with pretransplant DSAs. These findings, although novel in the context of pretransplant peripheral blood analysis in KTRs, align with previous posttransplant studies. For instance, Fouza et al and Fischman et al reported increased memory B-cell frequencies and decreased naive B cells at the time of DSA detection or shortly thereafter in posttransplant KTRs.[38,39] Although not reaching statistical significance, these trends suggest a potential contribution of the shift in B-cell subset distribution to the production of pretransplant DSAs.

Our analysis found no significant differences in immune checkpoint expression on B cells and subsets between KTRs with and without pretransplant DSAs or biopsy-proven rejection, which contradicts previous studies linking them to better transplant outcomes.[16,40] This discrepancy may arise from differences in study design, insufficient statistical power, or the complex nature of immune regulation in transplantation. Similarly, our examination of B-cell cytokine production revealed no significant differences in the frequencies of proinflammatory (IL-6 and TNF-α) or anti-inflammatory (IL-10) cytokines among groups. This contrasts with prior research

associating higher IL-10-producing B cells with better outcome and vice versa.[15] It is important to note that our analysis was broader (we considered the entire $CD19^{+}CD20^{+/-}$ B-cell population) rather than focusing solely on Bregs, which could explain the differences in findings.

Although our study, which combines spectral flow cytometry with functional cytokine production assays, provided novel insights into B cells, pretransplant DSAs, and rejection in KTRs, it was not without limitations. The retrospective design might have introduced sampling biases, emphasizing the need for validation through a prospective cohort study. Additionally, the study results may be influenced by its reliance on materials from an earlier era of kidney transplantation. Although we found consistent association between resting memory B cells and rejection across older and newer transplantation eras, advancements in transplantation protocols, including more sensitive risk assessment methods and improved immunosuppressive medications, have occurred since then that could potentially have altered the likelihood of rejection occurrence in more recent transplant cases. For instance, the unavailability of Luminex SAB at the time prevented pretransplant DSA detection, potentially affecting rejection risk assessment. Moreover, the prevalent use of cyclosporine instead of the more effective tacrolimus for maintenance immunosuppression may have impacted rejection rates. The cross-sectional nature of the study also limited our ability to observe changes in B-cell populations over time or examine the impact of immunosuppressive medications on our findings. These factors should be considered when interpreting the results and their applicability to current transplantation practices. In addition, the influence of HLA epitope mismatch was not examined, especially with regard to the generation of de novo DSAs, as we lacked posttransplant materials for examination. Evidence from published studies indeed suggests that high epitope mismatch, particularly for class II HLA, increases immune sensitivity to donor HLA antigens, potentially leading to alloimmune responses. This increased sensitivity can result in the activation of memory B cells and the production of DSAs.[41,42] Therefore, we could not rule out this effect and its potential influence on the occurrence of rejection in this cohort. Furthermore, although we examined cytokine production, more extensive functional assays, such as assessing the ability of resting memory B cells to produce HLA antibodies including DSAs, could deepen our understanding of their role in rejection. These limitations offer opportunities for future research which could provide more robust and broader insights into the role of B cells in transplant outcomes.

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REFERENCES

1. Schmitz R, Fitch ZW, Schroder PM, et al. B cells in transplant tolerance and rejection: friends or foes? *Transplant Inter*. 2020;33:30–40.
2. Anwar IJ, DeLaura IF, Gao Q, et al. Harnessing the B cell response in kidney transplantation—current state and future directions. *Front Immunol*. 2022;13. doi: 10.3389/ fimmu.2022.903068.
3. Louis K, Bailly E, Macedo C, et al. T-bet+CD27+CD21– B cells poised for plasma cell differentiation during antibody-mediated rejection of kidney transplants. *JCI Insight*. 2021;6. doi: 10.1172/ jci.insight.148881.
4. Shabir S, Girdlestone J, Briggs D, et al. Transitional B lymphocytes are associated with protection from kidney allograft rejection: a prospective study. *Am J Transplant*. 2015;15:1384–1391.
5. Ding T, Yan F, Cao S, et al. Regulatory B cell: new member of immunosuppressive cell club. *Hum Immunol*. 2015;76:615–621.
6. Kessel A, Haj T, Peri R, et al. Human CD19+CD25high B regulatory cells suppress proliferation of CD4+ T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. *Autoimmun Rev*. 2012;11:670–677.
7. Chesneau M, Michel L, Dugast E, et al. Tolerant kidney transplant patients produce B cells with regulatory properties. *J Am Soc Nephrol*. 2015;26:2588–2598.
8. Nouël A, Simon Q, Jamin C, et al. Regulatory B cells: an exciting target for future therapeutics in transplantation. *Front Immunol*. 2014;5:11.
9. Blair PA, Noreña LY, Flores-Borja F, et al. CD19+CD24hiCD38hi B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. *Immunity*. 2010;32:129–140.
10. Floudas A, Amu S, Fallon PG. New insights into IL-10 dependent and IL-10 independent mechanisms of regulatory B cell immune suppression. *J Clin Immunol*. 2016;36:25–33.
11. Iwata Y, Matsushita T, Horikawa M, et al. Characterization of a rare IL-10–competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood*. 2011;117:530–541.
12. 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.
13. Heidt S, Hester J, Shankar S, et al. B cell repopulation after alemtuzumab induction—transient increase in transitional B cells and long-term dominance of naïve B cells. *Am J transplant*. 2012;12:1784–1792.
14. Schlößer HA, Thelen M, Dieplinger G, et al. Prospective analyses of circulating B cell subsets in ABO-compatible and ABO-incompatible kidney transplant recipients. *Am J transplant*. 2017;17:542–550.
15. Cherukuri A, Rothstein DM, Clark B, et al. Immunologic human renal allograft injury associates with an altered IL-10/TNF-α expression ratio in regulatory B cells. *J Am Soc Nephrol*. 2014;25:1575–1585.
16. Irure-Ventura J, San Segundo D, Rodrigo E, et al. High pretransplant BAFF levels and B-cell subset polarized towards a memory phenotype as predictive biomarkers for antibody-mediated rejection. *Int J Mol Sci* . 2020;21:779.
17. Wehmeier C, Karahan GE, Krop J, et al; Swiss Transplant Cohort Study. Donor-specific B cell memory in alloimmunized kidney transplant recipients: first clinical application of a novel method. *Transplantation*. 2020;104:1026–1032.
18. Altulea D, van den Born JC, Diepstra A, et al. Memory B-cell derived donor-specific antibodies do not predict outcome in sensitized kidney transplant recipients: a retrospective single-center study. *Front Immunol*. 2024;15:1360627.
19. Peng B, Ming Y, Yang C. Regulatory B cells: the cutting edge of immune tolerance in kidney transplantation. *Cell Death Dis*. 2018;9:109.
20. van der Zwan M, Hesselink DA, van den Hoogen MWF, et al. Costimulation blockade in kidney transplant recipients. *Drugs*. 2020;80:33–46.
21. Garcia-Carmona Y, Ting AT, Radigan L, et al. TACI isoforms regulate ligand binding and receptor function. *Front Immunol*. 2018;9:2125.
22. Shi X, Que R, Liu B, et al. Role of IL-21 signaling pathway in transplant-related biology. *Transplant Rev*. 2016;30:27–30.
23. de Leur K, Dor FJMF, Dieterich M, et al. IL-21 receptor antagonist inhibits differentiation of B cells toward plasmablasts upon alloantigen stimulation. *Front Immunol*. 2017;8:00306.
24. Riella LV, Paterson AM, Sharpe AH, et al. Role of the PD-1 Pathway in the immune response. *Am J Transplant*. 2012;12:2575–2587.
25. Ning Z, Liu K, Xiong H. Roles of BTLA in immunity and immune disorders. *Front Immunol*. 2021;12:654960.
26. Otten HG, Joosten I, Allebes WA, et al. The PROCARE consortium: toward an improved allocation strategy for kidney allografts. *Transpl Immunol*. 2014;31:184–190.
27. Kamburova EG, Hoitsma A, Claas FH, et al; PROCARE Consortium. Results and reflections from the PROfiling Consortium on antibody

repertoire and effector functions in kidney transplantation: a mini-review. *HLA*. 2019;94:129–140.
28. Loupy A, Haas M, Roufosse C, et al. The Banff 2019 Kidney Meeting Report (I): updates on and clarification of criteria for T cell– and antibody-mediated rejection. *Am J Transplant*. 2020;20:2318–2331.
29. Bonasia CG, Inrueangsri N, Bijma T, et al. Circulating B cells display differential immune regulatory molecule expression in granulomatosis with polyangiitis. *Clin Exp Immunol*. 2025;219. doi: 10.1093/cei/uxae096
30. Sanz I, Wei C, Jenks SA, et al. Challenges and opportunities for consistent classification of human B cell and plasma cell populations. *Front Immunol*. 2019;10. doi:10.3389/fimmu.2019.02458.
31. Graver JC, Jiemy WF, Altulea DHA, et al. Cytokine producing B-cells and their capability to polarize macrophages in giant cell arteritis. *J Autoimmun*. 2023;140:103111.
32. Chong AS, Ansari MJ. Heterogeneity of memory B cells. *Am J Transplant*. 2018;18:779–784.
33. Das A, Xu H, Wang X, et al. Double-positive CD21+CD27+ B cells are highly proliferating memory cells and their distribution differs in mucosal and peripheral tissues. *PLoS One*. 2011;6:e16524.
34. Yue W, Liu J, Li X, et al. Memory B cells and long-lived plasma cells in AMR. *Ren Fail*. 2022;44:1605–1615.
35. Svachova V, Sekerkova A, Hruba P, et al. Dynamic changes of B-cell compartments in kidney transplantation: lack of transitional B cells is associated with allograft rejection. *Transplant Inter*. 2016;29:540–548.
36. Perezpayá I, Garcia SG, Clos-Sansalvador M, et al. Molecular screening of transitional B cells as a prognostic marker of improved graft outcome and reduced rejection risk in kidney transplant. *Front Immunol*. 2024;15:1433832.
37. Cherukuri A, Salama AD, Carter CR, et al. Reduced human transitional B cell T1/T2 ratio is associated with subsequent deterioration in renal allograft function. *Kidney Int*. 2017;91:183–195.
38. Fischman C, Fribourg M, Fabrizio G, et al. Circulating B cells with memory and antibody-secreting phenotypes are detectable in pediatric kidney transplant recipients before the development of antibody-mediated rejection. *Transplant Direct*. 2019;5:e481.
39. Fouza A, Tagkouta A, Daoudaki M, et al. Exploring perturbations in peripheral B cell memory subpopulations early after kidney transplantation using unsupervised machine learning. *J Clin Med*. 2023;12:6331.
40. Guo L, Lv J, Zhang J, et al. Elevated serum IL-21 levels are associated with stable immune status in kidney transplant recipients and a mouse model of kidney transplantation. *Aging (Milano)*. 2020;12:18396–18414.
41. Bezstarosti S, Kramer CSM, Claas FHJ, et al. Implementation of molecular matching in transplantation requires further characterization of both immunogenicity and antigenicity of individual HLA epitopes. *Hum Immunol*. 2022;83:256–263.
42. Wiebe C, Nevins TE, Robiner WN, et al. The synergistic effect of class II HLA epitope-mismatch and nonadherence on acute rejection and graft survival. *Am J transplant*. 2015;15:2197–2202.