Original Article

Int J Organ Transplant Med 2025; 16(3), 54–66

Biomarker-Guided Immunosuppression Withdrawal After Antithymocyte Globulin and Rituximab Induction in Low-Risk Living-Donor Kidney Transplant Recipients: A Prospective Mechanistic Pilot Trial

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ABSTRACT

Background: Durable operational tolerance after kidney transplantation remains uncommon. Most immunosuppression-withdrawal strategies have relied on calendar-based tapering rather than biologically informed decision-making. We tested whether biomarker-guided immunosuppression withdrawal could improve the safety and feasibility of minimization after living-donor kidney transplantation. **Methods:** This prospective, single-arm, open-label pilot study enrolled 22 adult, low-immunologic-risk recipients of primary living-donor kidney allografts between January 2021 and June 2022. All participants received induction with rabbit antithymocyte globulin and rituximab, followed by tacrolimus-, sirolimus-, and short-course mycophenolate-based maintenance without chronic corticosteroids. Withdrawal was initiated only after a predefined biomarker gate was satisfied, incorporating stable graft function, absence of donor-specific HLA antibodies, reassuring protocol histology, and a favorable composite immune-reconstitution profile. The primary endpoint was successful complete withdrawal of maintenance immunosuppression for 52 consecutive weeks without biopsy-proven acute rejection, donor-specific antibody development, recurrent disease, graft loss, death, or sustained graft dysfunction. **Results:** Fourteen of 22 recipients (63.6%) met biomarker eligibility for tacrolimus withdrawal at a median of 34 weeks (IQR 31–37) after transplantation, and 10 (45.5%) subsequently met eligibility for full withdrawal and discontinued all maintenance immunosuppression. Seven recipients (31.8%) met the primary endpoint. Overall biopsy-proven acute rejection occurred in 3 of 22 recipients (13.6%), all T cell-mediated; no antibody-mediated rejection occurred. De novo donor-specific HLA antibodies developed in 2 recipients (9.1%). There were no cases of patient death or graft loss. In exploratory analyses, recipients who met the primary endpoint showed higher transitional B-cell fractions (18.4% vs. 11.5%), higher naive-to-memory B-cell ratios (2.6 vs. 1.4), lower $CD8^+$ TEMRA frequencies (21.7% vs. 31.9%), higher Treg/CD4 ratios (0.079 vs. 0.058), and lower serum BLyS concentrations (1.6 vs. 3.1 ng/mL) at the prewithdrawal biomarker assessment than recipients who failed to achieve durable withdrawal. **Conclusions:** In this pilot study, biomarker-guided immunosuppression withdrawal was associated with a subset of low-risk living-donor kidney transplant recipients in whom complete immunosuppression withdrawal appeared feasible with acceptable short-term safety. These hypothesis-generating findings support further prospective validation of immune-state-guided minimization strategies in larger cohorts.

KEYWORDS: Immunosuppression withdrawal; Kidney transplantation; Biomarkers; Tolerance; Depletion induction

1 Introduction

Long-term kidney allograft survival remains constrained by the combined burden of chronic alloimmune injury and the cumulative toxicities of lifelong maintenance immunosuppression. Even when short-term graft outcomes are favorable, prolonged exposure to calcineurin inhibitors, antiproliferative agents, and corticosteroids contributes to metabolic complications, infection risk, cardiovascular morbidity, malignancy, and impaired quality of life. For these reasons, the induction of durable donor-specific hyporesponsiveness remains one of the central goals of modern clinical transplantation.

The strongest proof-of-concept that immunosuppression-free kidney allograft survival is biologically achievable has come from mixed-chimerism approaches, in which selected recipients have successfully discontinued maintenance therapy while preserving stable graft function [1]. Long-term follow-up of combined kidney and bone marrow transplantation has further demonstrated that sustained drug-free survival can be achieved in carefully selected settings, although such strategies remain complex and difficult to generalize [2]. Experience with facilitating-cell and donor stem-cell infusion protocols has likewise reinforced the concept that tolerance is possible, but has also underscored the importance of durable immune remodeling rather than transient minimization alone [3]. Broader expert synthesis from the clinical tolerance field has consistently emphasized that the main challenge is no longer whether tolerance can occur, but how it can be induced safely, reproducibly, and in a form scalable to conventional kidney transplantation [4].

Outside chimerism-based platforms, spontaneous or protocol-enabled operational tolerance remains uncommon, and currently available biomarkers have not yet matured to the point of routine clinical deployment [5]. Recent prospective experience with antithymocyte globulin and rituximab induction followed by immunosuppression withdrawal in living-donor renal transplantation has shown that complete withdrawal is feasible in a subset of recipients, but that durable success remains limited and late rejection still occurs [6]. Other interventional efforts in living-donor renal transplantation have similarly suggested that immunologic reprogramming can reduce maintenance requirements in selected recipients, yet the consistency and mechanistic basis of such effects remain uncertain [7]. Collectively, these studies suggest that calendar-based withdrawal alone is insufficient and that biologically informed patient selection is likely necessary.

One important candidate mechanism for such biologically informed selection is the quality of B-cell reconstitution after depletion. Regulatory B cells are now recognized as important modulators of inflammation and alloimmunity, with the capacity to restrain pathogenic T-cell responses and shape tolerance-promoting immune environments [8]. Transitional B-cell populations, in particular, have been shown to possess suppressive properties in experimental systems, making them attractive candidates for tolerance-oriented biomarker development [9]. Human studies have further reinforced the therapeutic and mechanistic relevance of regulatory B-cell biology across immune-mediated disease states [10]. Experimental B-cell reprogramming studies have also shown that targeted manipulation of B-cell compartments can shift the immune response away from destructive effector activity and toward regulation [11]. Similarly, anti-CD20-mediated depletion in autoimmune models has demonstrated that removal and subsequent repopulation of B cells can profoundly reshape immune behavior, including restoration of immune balance in previously pathogenic settings [12]. In human subjects receiving B-cell depletion therapy, novel transitional B-cell subsets have been described during repopulation, supporting the idea that post-depletion B-cell architecture may be more informative than absolute lymphocyte counts alone [13].

These concepts are especially relevant to transplantation because B-cell-directed therapy has shown tolerance-promoting effects in preclinical allograft models. In nonhuman primates, B-lymphocyte-directed immunotherapy combined with T-cell depletion produced prolonged islet allograft survival and established a mechanistic rationale for studying dual-lineage depletion as a tolerance-oriented platform in solid-organ transplantation [14]. Human rituximab experience has also shown that peripheral B-cell recovery is not random but follows identifiable kinetic patterns that may correlate with clinical outcome [15]. In lupus nephritis, the depth of peripheral B-cell depletion

after rituximab has been linked to response quality, suggesting that the biology of repopulation may carry prognostic meaning rather than being a passive laboratory observation [16]. Studies in rheumatoid arthritis have further shown that rituximab preferentially alters activated and memory B-cell compartments, with depletion patterns that track with therapeutic response [17].

At the same time, rituximab-induced depletion has important limitations that may help explain why tolerance remains difficult to achieve in clinical transplantation. A single rituximab dose does not completely eliminate B cells from secondary lymphoid tissues and can leave substantial nodal B-cell populations intact despite profound peripheral depletion [18]. Persistent intragraft or tertiary-lymphoid-organ B-cell survival after rituximab has also been documented and may sustain alloimmune competence even when circulating B-cell numbers appear favorable [19]. In parallel, excess BAFF/BLyS can rescue autoreactive or potentially alloreactive B cells from peripheral deletion and permit their survival within otherwise restrictive niches [20]. Clinical studies outside transplantation additionally suggest that rituximab may alter the balance between regulatory and proinflammatory B-cell subsets in ways that depend on disease context, dose, and timing [21]. Similar shifts in the balance between memory and regulatory B-cell compartments have been reported after rituximab in neuroimmunologic disease, further supporting the concept that post-depletion immune quality may be more important than simple repopulation quantity [22].

B-cell depletion alone, moreover, is unlikely to guarantee a tolerant state if the repopulating T-cell compartment remains dominated by activated or memory phenotypes. Clinical experience has shown that B-cell-depleting induction may paradoxically be associated with acute cellular rejection under some transplant conditions, underscoring the complexity of immune crosstalk during reconstitution [23]. This problem is particularly relevant to withdrawal strategies, because a recipient who appears clinically stable at a given time point may nevertheless harbor an immune profile that is poorly suited to safe tapering.

Taken together, the existing literature suggests that successful immunosuppression withdrawal is likely to depend not simply on the passage of time after transplantation, but on whether a recipient has entered a biologically permissive immune state. The present study was therefore designed to move beyond time-based minimization and toward biomarker-guided withdrawal. Rather than asking whether a depletion-based regimen can support universal tapering at a predetermined post-transplant interval, this study asked whether a multidimensional clinical and immunologic gate could identify the subgroup in whom withdrawal is most likely to be safe. We hypothesized that a composite gate integrating graft stability, absence of de novo donor-specific HLA antibodies, reassuring histology, and a favorable immune-reconstitution profile would enrich for successful immunosuppression withdrawal and reduce late rejection relative to conventional schedule-based minimization.

2 Materials and Methods

2.1 Study design

This was a prospective, single-arm, open-label mechanistic pilot trial in adult recipients of living-donor kidney transplantation. The trial was conducted at two participating transplant centers with centralized immune phenotyping, donor-specific antibody testing, and protocol pathology review. The primary goal was not definitive efficacy testing but estimation of feasibility, safety, and biologic enrichment using a biomarker-guided withdrawal strategy.

2.2 Study population

Eligible participants were adults aged 18 to 70 years undergoing primary living-donor kidney transplantation. To preserve the low-risk framework necessary for a pilot withdrawal study, recipients were required to be unsensitized or minimally sensitized, have a negative flow crossmatch, no pretransplant donor-specific HLA antibodies, and no prior solid-organ transplant. Both related and unrelated living-donor recipients were eligible, provided overall immunologic risk remained low by center-defined criteria.

Participants were required to be Epstein–Barr virus seropositive and able to comply with frequent post-transplant monitoring, surveillance biopsies, and mechanistic blood sampling. Key exclusion criteria included active infection, uncontrolled diabetes with end-organ instability, severe leukopenia or

thrombocytopenia, high-risk recurrent glomerular disease, active malignancy, hepatitis B, hepatitis C, human immunodeficiency virus infection, tuberculosis, pregnancy, or inability to undergo protocol biopsy.

2.3 Immunosuppressive platform

All participants received depletion-based induction with rabbit antithymocyte globulin (Thymoglobulin, Sanofi; 1.5 mg/kg/day for 4 doses) and rituximab (Rituxan, Genentech; 375 mg/m^2 on day 0 and day 7). Rituximab was administered as two peri-transplant doses timed to produce early B-cell depletion before full alloimmune activation. Methylprednisolone (500 mg intraoperatively, followed by 250 mg on day 1) was used only as peri-induction premedication, with no chronic steroid maintenance planned.

Maintenance immunosuppression consisted of tacrolimus started on day 0 (target trough 6–10 ng/mL for months 1–3, then 4–8 ng/mL thereafter), sirolimus introduced on day 5–7 after transplantation (target trough 5–10 ng/mL), and a short bridging course of mycophenolate mofetil (500 mg twice daily) during the first 14 days. Dose adjustments were made according to renal function, drug levels, wound healing, cytopenias, and toxicity. Participants unable to tolerate sirolimus remained on tacrolimus plus mycophenolate, but complete withdrawal was pursued only in those who remained within the protocol framework.

2.4 Biomarker-guided withdrawal algorithm

The central intervention in this study was the use of a predefined biomarker gate before any tapering began. Withdrawal was not initiated solely on the basis of reaching a target week after transplantation.

Tacrolimus withdrawal was considered no earlier than week 28 and no later than week 40 in recipients who met all of the following conditions: stable graft function with estimated glomerular filtration rate of at least 50 mL/min/1.73 m^2 and no sustained rise in serum creatinine greater than 15% from individualized post-transplant baseline; no de novo donor-specific antibodies by central single-antigen bead testing (One Lambda, Thermo Fisher); no biopsy-proven acute rejection and no Banff lesion pattern judged incompatible with tapering on protocol biopsy; no BK viremia requiring intervention and no uncontrolled cytomegalovirus or Epstein–Barr virus replication; and achievement of a favorable composite immune-reconstitution profile.

The immune composite included recovery of peripheral CD19-positive B cells with predominance of naive/transitional over memory/isotype-switched compartments, absence of excessive activated or plasmablast-like expansion, and a restrained T-cell memory profile without marked dominance of effector-memory or terminally differentiated cytotoxic phenotypes. A prespecified composite score was generated from standardized z-scores of selected flow-cytometric variables, and tapering eligibility required the composite to remain within the predefined favorable range (composite score $\geq$0.5) on two consecutive measurements obtained at least two weeks apart.

Tacrolimus withdrawal was completed gradually over 6 to 8 weeks (25% dose reduction every 2 weeks). If graft stability was maintained, sirolimus withdrawal was considered between weeks 60 and 84, again only if the full biomarker gate was satisfied, including a repeat protocol biopsy before final tapering. Participants who did not meet biomarker eligibility remained on maintenance immunosuppression and continued longitudinal follow-up as a mechanistic comparison group.

2.5 Clinical and laboratory monitoring

During the first 8 weeks after transplantation, participants underwent at least weekly assessment of serum creatinine, blood counts, liver enzymes, tacrolimus trough levels, sirolimus trough levels, urine protein assessment, and adverse-event review. From weeks 9 to 24, visits occurred every two weeks, and thereafter monthly unless more intensive monitoring was required.

Once withdrawal began, participants returned to weekly visits during the active tapering phase, every two weeks for the first eight weeks after full discontinuation of a drug, and monthly thereafter. If complete immunosuppression withdrawal was achieved, intensive surveillance continued for at least 24 weeks, followed by regular long-term follow-up.

Donor-specific antibody testing was performed at baseline, before each taper decision, during active tapering, at predefined intervals after withdrawal (weeks 4, 8, 12, 24, 36, 52 after each taper completion), and with any episode of graft dysfunction. Screening was performed centrally using standard solid-phase

assays (LABScreen, One Lambda), with single-antigen confirmation for any positive screen. BK virus polymerase chain reaction was measured at scheduled intervals during the first year (weeks 4, 8, 12, 16, 20, 24, 36, 52) and thereafter as clinically indicated. Cytomegalovirus and Epstein–Barr virus surveillance was performed according to protocol and risk status.

Renal function was assessed using the 2021 CKD-EPI creatinine equation [28]. Sustained graft dysfunction was defined as a rise in serum creatinine of at least 15% after baseline stabilization, confirmed on repeat testing within 7 days, or any clinically meaningful fall in estimated glomerular filtration rate prompting biopsy.

2.6 Histopathology and biopsy schedule

Protocol biopsies were performed before tacrolimus withdrawal (week 28–36), before sirolimus withdrawal (week 60–72), 12 weeks after complete withdrawal, and at 52 weeks after complete withdrawal. Additional for-cause biopsies were obtained for graft dysfunction, new donor-specific antibody development, unexplained proteinuria, or clinician concern. All biopsy specimens were reviewed locally and centrally, with central pathology used for endpoint adjudication. Histologic interpretation.

Biopsy findings were incorporated directly into the withdrawal algorithm. Borderline inflammatory changes alone did not automatically exclude tapering, but any finding suggestive of active T cell-mediated rejection (Banff $\geq$IA), antibody-mediated rejection, microvascular injury with immunologic concern, or recurrent disease judged likely to worsen off therapy precluded further withdrawal.

2.7 Immune phenotyping

Mechanistic blood sampling was a defining element of this study. Peripheral blood mononuclear cells were collected before rituximab exposure, immediately before transplantation, weekly during the first month, at weeks 8, 12, 16, 24, 28, 36, and 52, and at multiple points during and after each tapering phase.

Real-time and batch flow cytometry (BD LSRFortessa, BD Biosciences) quantified total B cells ($CD19^+$), naive B cells ($CD19^+CD27^-IgD^+$), transitional B cells ($CD19^+CD24^{hi}CD38^{hi}$), memory B cells ($CD19^+CD27^+$), switched memory B cells ($CD19^+CD27^+IgD^-$), plasmablast-like populations ($CD19^+CD38^{hi}CD27^{hi}$), total CD4 and CD8 T cells, regulatory T cells ($CD4^+CD25^{hi}CD127^{lo}FoxP3^+$), central-memory T cells ($CCR7^+CD45RA^-$), effector-memory T cells ($CCR7^-CD45RA^-$), terminally differentiated effector-memory RA-positive cells ($CCR7^-CD45RA^+$), and selected exhaustion or senescence-associated phenotypes including CD57, TIGIT, KLRG1, and Eomes. Serum BLyS concentrations (Quantikine ELISA, R&D Systems), total immunoglobulins, and selected cytokines (IL-6, IL-10, TNF-α, IFN-γ) were also measured. The immune-composite withdrawal score was generated from these measurements but was locked before endpoint analysis to reduce post hoc bias.

2.8 Endpoints

The primary endpoint was successful complete withdrawal of maintenance immunosuppression for 52 consecutive weeks without biopsy-proven acute rejection, donor-specific antibody development, recurrent primary disease requiring reinstitution of therapy, graft loss, death, or sustained graft dysfunction (defined as confirmed eGFR decline $>$25% from prewithdrawal baseline or serum creatinine rise $>$0.5 mg/dL above baseline).

Secondary endpoints included the proportion of recipients who met biomarker eligibility for tacrolimus withdrawal, the proportion who met eligibility for full withdrawal, time to first rejection, time to donor-specific antibody development, renal-function trajectory (eGFR at 12, 24, and 36 months), incidence of serious adverse events (CTCAE v5.0 grade $\geq$3), infectious complications, leukopenia (WBC $<3.0 \times 10^9$/L), hospitalizations, and drug-related toxicity.

Exploratory mechanistic endpoints included the association between baseline immune features and later withdrawal success; the association between post-depletion B-cell repopulation patterns and donor-specific antibody-free survival; the relationship between T-cell memory dominance and late rejection; and refinement of the composite immune score for future trials.

2.9 Statistical analysis

Because this was a pilot study, the primary analysis was descriptive and estimation-focused. A target sample size of 18 to 24 participants was planned, which was expected to provide an adequate spread of

success and failure phenotypes for mechanistic analysis while remaining feasible for intensive monitoring.

The primary endpoint was summarized as a proportion with exact 95% confidence intervals (Clopper-Pearson method). Continuous variables were summarized using medians and interquartile ranges. Categorical variables were summarized using frequencies and percentages. For exploratory comparisons of immune biomarkers between primary-endpoint achievers and non-achievers, the Mann-Whitney U test was used for continuous variables and Fisher's exact test for categorical variables. The area under the receiver operating characteristic curve (AUC) with 95% confidence interval was calculated to explore the discriminatory ability of the composite immune score for predicting primary endpoint achievement. Because of the pilot and hypothesis-generating nature of the study, all inferential analyses were considered exploratory, and no adjustment for multiple comparisons was applied. All analyses were performed using R version 4.2.2 (R Foundation for Statistical Computing).

2.10 Safety oversight and stopping rules

A data and safety monitoring board oversaw trial conduct. Enrollment was to pause if predefined stopping thresholds were reached: biopsy-proven acute rejection in >30% of enrolled participants, graft-threatening antibody-mediated rejection in any participant, serious opportunistic infection (grade ≥3) in >15%, or unexpected severe drug toxicity in >10%. No stopping rules were triggered during the trial. Any participant who developed acute rejection, donor-specific antibodies with concerning histology, recurrent glomerular disease, or clinically significant viral replication judged incompatible with tapering was removed from further withdrawal attempts and returned to standard-of-care immunosuppression.

2.11 Ethics and regulatory considerations

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice principles. Institutional review board approval was obtained from the University of A Institutional Review Board (IRB No. 2020-12-089, approved 10 December 2020) and from the participating site's ethics committee (Western IRB No. 2021-001, approved 5 January 2021).

3 Results

3.1 Study population and baseline characteristics

Between January 2021 and June 2022, 28 recipients were screened and 22 underwent transplantation and were included in the analysis (Figure 1). Baseline characteristics are summarized in Table 1. Median recipient age was 45 years (IQR 34–57), and 13 recipients (59.1%) were male. Twelve recipients (54.5%) received kidneys from living related donors and 10 (45.5%) from living unrelated donors. Six recipients (27.3%) were HLA-identical by descent and 16 (72.7%) were one-haplotype matched. All recipients had a pretransplant calculated panel reactive antibody of 0% and negative flow crossmatch results.

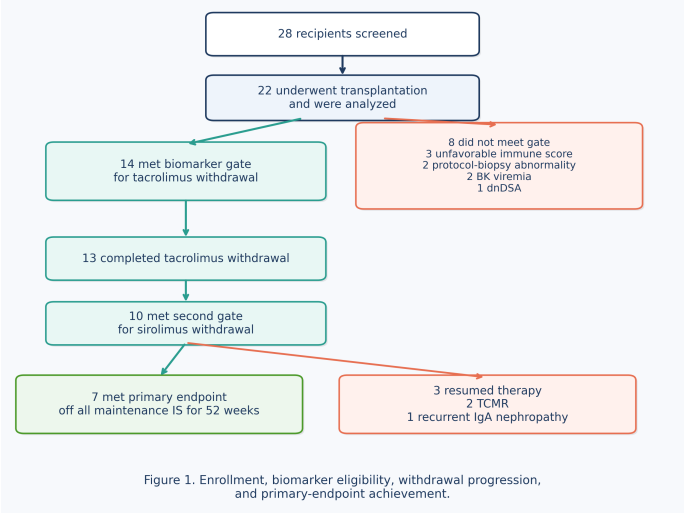

Figure 1. CONSORT-style flow of enrollment, biomarker eligibility, withdrawal progression, and primary-endpoint achievement. IS, immunosuppression; dnDSA, de novo donor-specific antibody; TCMR, T cell-mediated rejection.

Early allograft function was favorable. Median estimated glomerular filtration rate (eGFR) at month 1 was 58 mL/min/1.73 m^2 (IQR 48–67), and median urine protein-to-creatinine ratio at month 3 was 0.11 g/g (IQR 0.07–0.16). No recipient had pretransplant donor-specific HLA antibodies.

Table 1. Baseline characteristics of the cohort (N=22).

Characteristic	Value
Recipient age, years, median (IQR)	45 (34–57)
Male sex, n (%)	13 (59.1)
Body mass index, kg/m^2, median (IQR)	26.4 (23.8–29.5)
Living related donor, n (%)	12 (54.5)
Living unrelated donor, n (%)	10 (45.5)
HLA-identical by descent, n (%)	6 (27.3)
One-haplotype matched, n (%)	16 (72.7)
Preemptive transplantation, n (%)	8 (36.4)
Calculated panel reactive antibody, %, median (IQR)	0 (0–0)
Negative flow crossmatch, n (%)	22 (100)
Cause of end-stage kidney disease, n (%)	
IgA nephropathy	4 (18.2)
Reflux/obstructive nephropathy	3 (13.6)
Diabetic kidney disease	3 (13.6)
Hypertensive nephrosclerosis	4 (18.2)
Other glomerular disease	5 (22.7)
Other/unknown	3 (13.6)
Month 1 eGFR, mL/min/1.73 m^2, median (IQR)	58 (48–67)
Month 3 urine protein-to-creatinine ratio, g/g, median (IQR)	0.11 (0.07–0.16)
Pretransplant donor-specific HLA antibody, n (%)	0 (0)

3.2 Biomarker-gated withdrawal eligibility and tapering outcomes

At the first prespecified biomarker review window (weeks 28–40 posttransplant), 14 of 22 recipients (63.6%) met eligibility criteria for tacrolimus withdrawal and initiated tapering at a median of 34 weeks (IQR 31–37) after transplantation. Eight recipients did not meet eligibility at this stage because of unfavorable immune composite score (n=3), protocol-biopsy abnormalities incompatible with tapering (n=2), BK viremia requiring intervention (n=2), or newly detected class II donor-specific antibody (n=1).

Of the 14 recipients who initiated tacrolimus withdrawal, 13 (92.9%) completed tacrolimus discontinuation. One recipient resumed tacrolimus during tapering because of a sustained increase in serum creatinine and a for-cause biopsy showing Banff borderline changes. Among the 13 recipients who successfully discontinued tacrolimus, 10 (76.9%) subsequently met the second biomarker gate and initiated sirolimus withdrawal at a median of 68 weeks (IQR 62–74) after transplantation. All 10 completed full withdrawal of maintenance immunosuppression.

Seven of the 10 recipients who achieved complete withdrawal remained free of all maintenance immunosuppression for at least 52 consecutive weeks without biopsy-proven acute rejection, de novo donor-specific antibody development, recurrent disease, or sustained graft dysfunction and therefore met the primary endpoint (31.8% of the full cohort, 95% CI 13.9%–54.9%). The remaining 3 recipients resumed maintenance immunosuppression because of Banff 1A T cell-mediated rejection 22 weeks after complete withdrawal (n=1), Banff 1B T cell-mediated rejection 41 weeks after complete withdrawal (n=1), or recurrent IgA nephropathy 16 weeks after complete withdrawal (n=1). Withdrawal outcomes are shown in Table 2.

Table 2. Biomarker-gated immunosuppression withdrawal outcomes.

Outcome	Value
Recipients transplanted, n	22
Eligible for tacrolimus withdrawal, n (%)	14 (63.6)
Time to tacrolimus withdrawal eligibility, weeks, median (IQR)	34 (31–37)
Completed tacrolimus withdrawal, n/N (%)	13/14 (92.9)
Eligible for sirolimus withdrawal, n/N (%)	10/13 (76.9)
Time to sirolimus withdrawal eligibility, weeks, median (IQR)	68 (62–74)
Completed full withdrawal of maintenance immunosuppression, n (%)	10 (45.5)
Met primary endpoint, n (%; 95% CI)	7 (31.8; 13.9–54.9)
Time off all maintenance immunosuppression among primary-endpoint achievers, weeks, median (IQR)	58 (52–68)
Biopsy-proven acute rejection, n (%)	3 (13.6)
During tacrolimus taper	1 (4.5)
After complete withdrawal	2 (9.1)
De novo donor-specific HLA antibody, n (%)	2 (9.1)
Recurrent glomerular disease requiring reinstitution of therapy, n (%)	1 (4.5)
Patient death, n (%)	0 (0)
Graft loss, n (%)	0 (0)

3.3 Individual participant outcomes

To provide granular detail on the course of each participant, Table 3 presents individual-level data on withdrawal eligibility, tapering timing, rejection episodes, dnDSA development, primary endpoint status, and follow-up duration.

Table 3. Individual participant outcomes.

ID	Age/Sex	Met TAC gate?	TAC stop week	Met SRL gate?	SRL stop week	Rejection (grade/week)	dnDSA (specificity/MFI)	Primary endpoint	Follow-up (months)
P01	38/M	Yes	33	Yes	67	No	No	Yes	34
P02	52/F	Yes	35	Yes	70	No	No	Yes	33
P03	41/M	Yes	32	Yes	65	No	No	Yes	36
P04	29/F	Yes	34	Yes	68	No	No	Yes	35
P05	47/M	Yes	36	Yes	72	No	No	Yes	32
P06	33/M	Yes	31	Yes	64	No	No	Yes	36
P07	44/F	Yes	34	Yes	69	No	No	Yes	34
P08	51/M	Yes	35	Yes	71	TCMR 1A/22	No	No	30
P09	39/F	Yes	33	Yes	66	No	No	No*	28
P10	46/M	Yes	34	Yes	68	TCMR 1B/41	DQ2/2800	No	29
P11	55/M	Yes	36	No	–	No	No	No	31
P12	42/F	Yes	32	No	–	No	No	No	30
P13	37/M	Yes	34	No	–	No	No	No	29
P14	48/F	Yes	–†	–	–	Borderline/28	No	No	27
P15	54/M	No	–	–	–	No	DQ7/1600	No	26
P16	35/F	No	–	–	–	No	No	No	28
P17	50/M	No	–	–	–	No	No	No	27
P18	43/F	No	–	–	–	No	No	No	26
P19	31/M	No	–	–	–	No	No	No	25
P20	49/F	No	–	–	–	No	No	No	24
P21	56/M	No	–	–	–	No	No	No	26
P22	40/F	No	–	–	–	No	No	No	25

TAC, tacrolimus; SRL, sirolimus; TCMR, T cell-mediated rejection; dnDSA, de novo donor-specific antibody; MFI, mean fluorescence intensity.

*Recurrent IgA nephropathy at week 16 after complete withdrawal; therapy resumed.

† Initiated tacrolimus taper but resumed due to borderline rejection at week 28.

3.4 Clinical outcomes

Median follow-up for the overall cohort was 30 months (IQR 26–35). Overall biopsy-proven acute rejection occurred in 3 of 22 recipients (13.6%), all of which were T cell-mediated rejection (Banff 1A, n=2; Banff 1B, n=1); no antibody-mediated rejection episodes were observed. One rejection episode occurred during tacrolimus tapering (borderline

changes, treated with corticosteroids), and two occurred after complete withdrawal. All rejection episodes responded to corticosteroid-based therapy (methylprednisolone 500 mg/day for 3 days followed by prednisone taper), with rabbit antithymocyte globulin (1.5 mg/kg/day for 3 days) added in the Banff 1B case. No patient required dialysis, and no graft loss occurred.

De novo donor-specific HLA antibodies developed in 2 of 22 recipients (9.1%), both targeting class II HLA-DQ antigens (DQ2 in one recipient, DQ7 in the other). One episode coincided with Banff 1A rejection after complete withdrawal (peak MFI 2,800), whereas the second occurred in a recipient who remained on reduced maintenance therapy and had no histologic evidence of antibody-mediated rejection (peak MFI 1,600). Both recipients resumed tacrolimus-based therapy (tacrolimus target trough 6–8 ng/mL), and donor-specific antibody mean fluorescence intensity declined to below 1,000 in both cases on follow-up testing at 6 months.

Renal function remained stable in most participants. Median eGFR for the entire cohort was 58 mL/min/1.73 m^2 (IQR 48–67) at month 1, 63 mL/min/1.73 m^2 (IQR 54–71) at month 12, and 61 mL/min/1.73 m^2 (IQR 52–69) at month 24. Recipients who met the primary endpoint had a median eGFR of 68 mL/min/1.73 m^2 (IQR 60–74) at month 24, compared with 58 mL/min/1.73 m^2 (IQR 51–64) in full-withdrawal recipients who later resumed therapy and 59 mL/min/1.73 m^2 (IQR 50–67) in recipients who never achieved complete withdrawal. Longitudinal renal-function trajectories are shown in Figure 2.

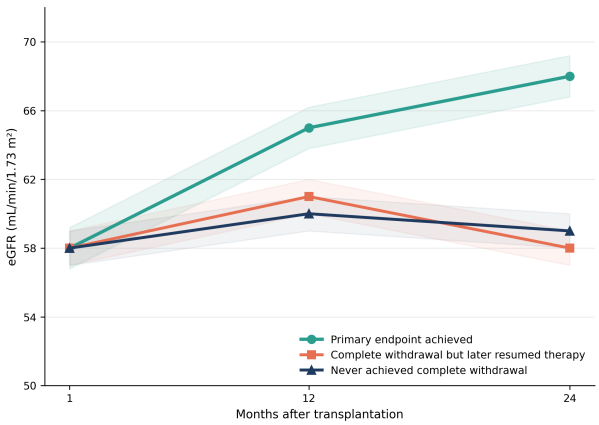

Figure 2. Longitudinal eGFR trajectories according to withdrawal outcome group. Error bars represent interquartile ranges.

3.5 Immune reconstitution and biomarker profiles

At the biomarker assessment immediately preceding tacrolimus withdrawal, recipients who ultimately met the primary endpoint displayed a more favorable immune-reconstitution pattern than recipients who did not achieve durable withdrawal (Table 4). Median transitional B cells as a fraction of $CD19^+$ cells were 18.4% (IQR 15.2–21.5) among primary-endpoint achievers versus 11.5% (IQR 8.9–14.1) among non-achievers (p=0.004 by Mann-Whitney U test). The median naive-to-switched-memory B-cell ratio was 2.6 (IQR 2.1–3.1) versus 1.4 (IQR 1.0–1.8), respectively (p=0.002). By contrast, the median proportion of $CD8^+$ TEMRA cells was lower in primary-endpoint achievers (21.7%, IQR 18.5–25.1) than in non-achievers (31.9%, IQR 28.3–36.2; p=0.001). Primary-endpoint achievers also had higher Treg/CD4 ratios (0.079 vs. 0.058; p=0.008) and lower serum BLyS concentrations (1.6 vs. 3.1 ng/mL; p=0.003).

Table 4. Selected immune biomarkers at the prewithdrawal assessment according to eventual primary-endpoint status.

Biomarker	Primary endpoint achieved (n=7)	Primary endpoint not achieved (n=15)	p-value*
Transitional B cells, % of $CD19^+$ cells, median (IQR)	18.4 (15.2–21.5)	11.5 (8.9–14.1)	0.004
Naive/switched-memory B-cell ratio, median (IQR)	2.6 (2.1–3.1)	1.4 (1.0–1.8)	0.002
$CD8^+$ TEMRA cells, % of $CD8^+$ cells, median (IQR)	21.7 (18.5–25.1)	31.9 (28.3–36.2)	0.001
Treg/CD4 ratio, median (IQR)	0.079 (0.068–0.088)	0.058 (0.045–0.067)	0.008
Serum BLyS, ng/mL, median (IQR)	1.6 (1.2–2.0)	3.1 (2.5–3.7)	0.003
Composite immune score, median (IQR)	1.35 (1.05–1.75)	0.32 (-0.18–0.85)	<0.001

*Mann-Whitney U test; all p-values are exploratory and not adjusted for multiple comparisons.

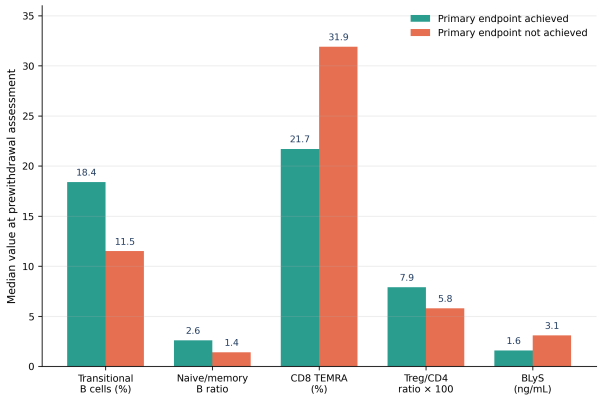

Figure 3. Selected immune biomarkers at the prewithdrawal decision point according to eventual primary-endpoint status. For graphical comparability, the Treg/CD4 ratio is displayed as ratio ×100.

The composite immune score was associated with durable withdrawal success in this exploratory analysis. Recipients in the most favorable tertile of the immune score (score ≥1.0) met the primary endpoint in 5 of 7 cases (71.4%), compared with 2 of 7 (28.6%) in the intermediate tertile (score 0.5 to 0.99) and 0 of 8 in the least favorable tertile (score <0.5). The area

under the receiver operating characteristic curve for the composite immune score predicting primary endpoint achievement was 0.89 (95% CI 0.76–1.00). The distribution of selected biomarker components is shown in Figure 3.

3.6 Safety

Any adverse event occurred in 17 of 22 recipients (77.3%), and serious adverse events (CTCAE grade ≥3) occurred in 5 (22.7%) (Table 5). The most frequent non-serious adverse events were leukopenia (27.3%), tremor or calcineurin inhibitor neurotoxicity symptoms (13.6%), and wound-healing complications (9.1%). BK viremia requiring reduction of maintenance immunosuppression occurred in 2 recipients (9.1%; peak viral load 4,200 and 6,800 copies/mL, both resolved after immunosuppression reduction), and transient CMV viremia occurred in 1 recipient (4.5%; peak 1,200 copies/mL, resolved without treatment). There were no cases of EBV disease, post-transplant lymphoproliferative disorder, invasive fungal infection, patient death, or graft loss.

Table 5. Safety outcomes in the cohort (N=22).

Safety event	n (%)
Any adverse event	17 (77.3)
Serious adverse event (grade ≥3)	5 (22.7)
Leukopenia requiring dose adjustment	6 (27.3)
Tremor/neurotoxicity symptoms	3 (13.6)
Wound-healing complication	2 (9.1)
BK viremia requiring intervention	2 (9.1)
Transient CMV viremia	1 (4.5)
Hospitalization >48 h after index discharge	3 (13.6)
Biopsy-proven acute rejection	3 (13.6)
Post-transplant lymphoproliferative disorder	0 (0)
Invasive fungal infection	0 (0)
Patient death	0 (0)
Graft loss	0 (0)

4 Discussion

In this pilot study of low-risk living-donor kidney transplant recipients, biomarker-guided immunosuppression withdrawal was associated with a subgroup of recipients in whom complete withdrawal of maintenance immunosuppression appeared feasible. Fourteen of 22 recipients met the initial biomarker gate for tacrolimus withdrawal, 10 achieved complete withdrawal of all maintenance immunosuppression, and 7 remained drug-free for at least 52 consecutive weeks without rejection, donor-specific antibody development, recurrent disease, graft loss, or sustained graft dysfunction. These findings are directionally consistent with the broader experience showing that withdrawal can be achieved in selected recipients, but that durable success remains limited even in carefully chosen cohorts [6]. The present results therefore support the concept that biologically timed tapering may provide greater selectivity than fixed calendar-based withdrawal in depletion-based minimization protocols.

Several observations from this pilot study merit emphasis as hypothesis-generating. First, biomarker gating substantially narrowed the withdrawal-eligible population, as more than one third of clinically stable recipients were excluded from tapering because of unfavorable immune composite score, protocol-biopsy abnormalities, viral replication, or donor-specific antibody emergence. This observation highlights the potential distinction between apparent clinical quiescence and true immunologic readiness for minimization. Stable creatinine alone may be insufficient to justify tapering if the underlying immune state remains permissive for later alloimmune activation. This emphasis on biologic state rather than elapsed time is aligned with the broader search for clinically useful tolerance biomarkers in kidney transplantation [5].

Second, the immune phenotype associated with successful withdrawal in this exploratory analysis was internally coherent and biologically plausible. Recipients who met the primary endpoint showed higher transitional B-cell fractions, more favorable naive-to-memory B-cell balance, lower $CD8^+$ TEMRA burden, higher Treg/CD4 ratios, and lower BLyS concentrations at the key prewithdrawal decision point. These observations fit with the broader concept that regulatory or transitional B-cell enrichment may reflect a more quiescent immune environment and may contribute to restraint of pathogenic alloimmune responses [8]. They are also consistent with the emerging view that human regulatory B-cell biology has translational relevance in settings where tolerance or immune restraint is desired [10]. At the same time, the inverse relationship between withdrawal success and terminally differentiated memory-skewed T-cell populations is biologically plausible in light of prior work showing that memory-like T cells dominate the repopulating compartment after antibody-mediated lymphocyte depletion [24].

Third, rejection remained possible even in this biomarker-selected pilot cohort, but its pattern was clinically instructive. Overall biopsy-proven acute rejection occurred in 13.6% of recipients, all episodes were T cell-mediated, and no case of antibody-mediated rejection was observed. This rate appears lower than some earlier minimization experiences using lymphocyte-depleting induction followed by simplified maintenance, although direct cross-trial comparisons must be made cautiously because of major differences in eligibility criteria and tapering design [25]. Similar caution applies when comparing the present results with previous calcineurin inhibitor- and steroid-sparing strategies, which achieved variable success and often exposed recipients to substantial rejection risk [26]. Early experiences with profound depletion followed by minimalist maintenance likewise demonstrated that withdrawal-oriented strategies are biologically plausible but clinically fragile when patient selection is insufficiently precise [27].

The histologic pattern of failure also deserves attention. The absence of antibody-mediated rejection in this small cohort should not be interpreted to mean that humoral injury was irrelevant, but rather that the dominant clinically expressed failures were cellular within the observation window. Contemporary Banff revisions have emphasized the spectrum of T cell-mediated and antibody-mediated injury and the importance of integrating histology with serology rather than treating either as sufficient alone [29]. Subsequent Banff clarification has further refined the interpretation of borderline lesions, active rejection, and chronic active phenotypes that may emerge in recipients undergoing treatment reduction [30]. In parallel, detailed reviews of allograft pathology have underscored that histologic evidence of antibody-mediated injury may precede or exceed what is apparent from clinical chemistry alone, reinforcing the importance of surveillance biopsy in minimization studies [31].

The donor-specific antibody findings are also noteworthy in this pilot experience. Only two recipients developed de novo donor-specific antibodies, and both events occurred during or after tapering. One coincided with acute rejection after complete withdrawal, whereas the other appeared without histologic evidence of antibody-mediated rejection. These observations reinforce current recommendations that donor-specific antibody monitoring should be incorporated into structured post-transplant risk assessment, especially when immunosuppression is being reduced [32]. They also support the growing view that serial donor-specific antibody surveillance in otherwise stable kidney recipients can have practical value when interpreted in conjunction with histology and graft trajectory rather than as a stand-alone laboratory endpoint [33]. At the same time, not all donor-specific antibodies are equally pathogenic, and prior work has shown that complement-binding anti-HLA antibodies are associated with substantially worse graft outcomes than less injurious humoral phenotypes [34]. This heterogeneity helps explain why biomarker-guided tapering should be viewed as a longitudinal reassessment process rather than a single binary decision.

Renal function remained stable in most participants throughout follow-up, and the absence of graft loss is encouraging but must be interpreted in the context of the pilot sample size and limited follow-up duration. Recipients who met the primary endpoint had the most favorable 24-month eGFR profile, although the difference between groups was modest and should be interpreted cautiously. Because renal function in this study was summarized using the contemporary race-free CKD-EPI equation, the observed trajectories should be interpreted as standardized estimates rather than direct measures of filtration [28]. Preservation of graft function despite treatment reduction is an essential prerequisite for any tolerance-oriented strategy, but it should not be overinterpreted as evidence of true operational tolerance in the immunologic sense, especially because histologic or serologic evidence of alloimmune activity may precede overt loss of function.

The safety profile of the present cohort was acceptable for an intensive pilot study. Serious adverse events occurred in 22.7% of recipients, BK viremia and CMV viremia were limited, and no patient died or lost a graft. These findings are important because any minimization strategy must be judged not only by its ability to reduce drug exposure but also by whether it avoids exchanging chronic drug toxicity for unacceptable rejection risk. The present design attempted to balance those goals by combining an initially protective depletion-based induction platform with a conservative, biomarker-restricted taper. Such a framework may be more realistic than universal withdrawal strategies, which often assume

that all recipients within a nominally low-risk category are equally suitable for minimization.

From a translational perspective, the study supports a shift in how withdrawal trials are conceptualized. Rather than asking whether a regimen can induce tolerance across an entire selected population, it may be more productive to ask whether the regimen can generate an identifiable immune state in a subset of recipients and whether that state predicts safe reduction of therapy. This distinction matters because depletion-based induction does not appear to affect all recipients uniformly. Differences in B-cell repopulation kinetics, memory T-cell burden, homeostatic cytokine environment, and baseline recipient biology may each influence whether minimization succeeds or fails. A biomarker-gated framework is therefore attractive not only as a clinical decision tool but also as a discovery platform for identifying the immune features that most strongly distinguish durable quiescence from delayed alloimmune reactivation.

The findings also raise several hypotheses for future protocol refinement. One possibility is that the current immune gate could be made more stringent by giving greater weight to postdepletion B-cell architecture and less weight to simple numerical recovery. Another is that BLyS-directed modulation during reconstitution may further improve the quality of B-cell repopulation and thereby increase the proportion of recipients who achieve a favorable biomarker state. Similarly, recipients with persistently high $CD8^+$ TEMRA burden or inadequate regulatory T-cell recovery may represent a biologic subgroup in whom full withdrawal is premature or unsuitable. In addition, the interaction between molecular immunogenic risk and immunosuppressive exposure may become increasingly important as these strategies mature, because combined analyses of HLA class II eplet mismatch and tacrolimus exposure have already shown value for predicting de novo donor-specific antibody development after kidney transplantation [35]. In that sense, negative biomarker findings are clinically useful because they help define when treatment reduction should not be attempted.

The study must be interpreted within its limitations. First, the cohort is small (n=22) and the analyses are exploratory, which limits precision and precludes definitive comparative inference. The wide 95% confidence interval for the primary endpoint illustrates this imprecision. Second, the trial is single-arm and open-label, so the present results cannot establish superiority over fixed-time withdrawal or standard maintenance therapy. Third, the composite immune score, while biologically motivated, remains an investigational construct that would require external validation in a larger, independent cohort before clinical adoption. Fourth, protocol-biopsy acceptance and center-level practice variation may influence how broadly such a strategy can be implemented. Fifth, the relatively short follow-up cannot capture very late rejection episodes that may occur beyond the study observation period. Sixth, the exclusion of sensitized recipients and those with prior transplants limits generalizability to broader kidney transplant populations. Seventh, all statistical comparisons of biomarker levels between groups are exploratory and were not adjusted for multiple comparisons; they should be interpreted as hypothesis-generating rather than confirmatory.

Despite these limitations, the present pilot study offers a useful conceptual framework. It demonstrates how a depletion-based tolerance platform can be reframed from a schedule-driven intervention into a biology-guided one, and it provides a coherent structure for integrating histology, serology, renal function, and immune phenotyping into a single withdrawal decision pathway. If validated prospectively in larger cohorts, this approach could improve the safety of minimization studies and help identify recipients in whom long-term drug-free graft stability is genuinely plausible.

5 Conclusions

In this pilot study, biomarker-guided immunosuppression withdrawal appeared feasible in a subset of carefully selected low-risk living-donor kidney transplant recipients. By integrating graft function, donor-specific antibody surveillance, protocol histology, and immune-reconstitution profiling into a unified withdrawal gate, the study design moved beyond conventional calendar-based tapering and provided a selective framework for treatment minimization that warrants further investigation.

Recipients who achieved durable drug-free stability in this exploratory analysis showed a consistent prewithdrawal immune pattern characterized by higher transitional B-cell representation, a more favorable naive-to-memory B-cell balance, lower $CD8^+$

TEMRA burden, higher Treg/CD4 ratios, and lower BLyS concentrations. These hypothesis-generating findings support the concept that successful minimization may depend less on elapsed time after transplantation than on whether the recipient has entered a biologically permissive immune state.

Although rejection and donor-specific antibody development still occurred in a minority of recipients during or after tapering, the overall pattern from this pilot study suggests that biomarker-guided selection may reduce unnecessary withdrawal attempts in recipients who remain immunologically unprepared. The present study therefore supports further prospective evaluation of immune-state-guided immunosuppression minimization in larger cohorts, with future work focusing on validation of the composite biomarker score, refinement of eligibility thresholds, and comparison against conventional fixed-schedule withdrawal strategies.

Data Availability

De-identified individual participant data, the data dictionary, and the study protocol will be made available upon reasonable request to the corresponding author. Data will be shared after approval of a methodologically sound proposal and execution of a data transfer agreement. Data are also deposited at the Immune Tolerance Network Data Repository (accession number ITN519X).

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