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Original Article

Immunosuppression withdrawal in living-donor renal transplant recipients following induction with antithymocyte globulin and rituximab: Results of a prospective clinical trial

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ABSTRACT

Durable tolerance in kidney transplant recipients remains an important but elusive goal. We hypothesized that adding B cell depletion to T cell depletion would generate an immune milieu postreconstitution dominated by immature transitional B cells, favoring tolerance. The Immune Tolerance Network ITN039ST Research Study of ATG and Rituximab in Renal Transplantation was a prospective multicenter pilot study of live donor kidney transplant recipients who received induction with rabbit antithymocyte globulin and rituximab and

Abbreviations: AE, adverse event; AMR, antibody-mediated rejection; ATG, antithymocyte globulin; BlyS, B lymphocyte stimulator; CMV, cytomegalovirus; DSA, donor-specific HLA antibody; EBV, Epstein-Barr virus; GFR, glomerular filtration rate; HLA, human leukocyte antigen; Ig, immunoglobulin; IS, Immunosuppression; ISW, immunosuppression withdrawal; IVIg, intravenous immunoglobulin; IV, intravenous; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; NCT, national clinical trial; NHP, nonhuman primate; NK, natural killer; PBMC, peripheral blood mononuclear cells; PRA, panel reactive antibody; PCR, polymerase chain reaction; RESTARRT, Research Study of ATG and Rituximab in Renal Transplantation; SAE, serious adverse event; SCr, serum creatinine; TCM, T central memory cells; TCMR, T cell-mediated rejection; TEM, T effector memory cells; TEMRA, T effector memory RA+ cells; Treg, regulatory T cells.

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globulin
rituximab

initiated immunosuppression (IS) withdrawal (ISW) at 26 weeks. The primary endpoint was freedom from rejection at 52 weeks post-ISW. Six of the 10 subjects successfully completed ISW. Of these 6 subjects, 4 restarted immunosuppressive medications due to acute rejection or recurrent disease, 1 remains IS-free for over 9 years, and 1 was lost to follow-up after being IS-free for 42 weeks. There were no cases of patient or graft loss. $CD19^+$ B cell frequencies returned to predepletion levels by 26 weeks posttransplant; immunoglobulin D^+CD27^--naïve B cells predominated. In contrast, memory cells dominated the repopulation of the T cell compartment. A regimen of combined B and T cell depletion did not generate the tolerogenic B cell profile observed in preclinical studies and did not lead to durable tolerance in the majority of kidney transplant recipients.

1. Introduction

Immunologic tolerance to an organ or tissue transplant is a long-sought-after goal of the transplant community. While successful tolerance has been achieved by induction of hematopoietic chimerism,[1-3] such protocols require intensive pretransplant conditioning that limits broad application to deceased donor kidneys and other organs and can result in life-threatening graft versus host disease.[4] These limitations prompt a continued search for more effective and well-tolerated regimens that can be initiated during the peritransplant period.

An attractive alternative is to capitalize on naturally occurring regulatory pathways essential to the generation and maintenance of self-tolerance. A number of studies suggest that B cells expressing regulatory activity may promote tolerance.[5-7] Spontaneously tolerant renal allograft recipients who had discontinued immunosuppressive medications due to noncompliance or medical necessity (such as malignancy or infection) were found to manifest a peripheral blood immune cell transcriptional profile dominated by B cell genes.[8] Equally provocative were mechanistic studies that correlated tolerance following B cell depletion with the repopulation of B cells exhibiting a shift from a predominantly mature/memory phenotype to an immature/naïve/-transitional phenotype.[9-11]

In an allogeneic pancreatic islet transplant study in nonhuman primates (NHP), addition of B cell targeting with rituximab to T cell depletion with antithymocyte globulin (ATG) followed by transient sirolimus maintenance resulted in long-term islet allograft survival; in one case, for years after discontinuation of maintenance IS.[12] Loss of tolerance, when it occurred, was temporally associated with a loss of the B cell population shift, noted above, and accumulation of mature B cells.

Based on these findings, we designed and conducted a prospective trial in well-matched recipients of a live donor renal transplant to test the hypothesis that induction with rituximab and ATG followed by standard maintenance immunosuppression (IS) would effectively prevent allograft rejection and allow staged weaning of IS. The rationale for this induction regimen was the observation that it led to a preponderance of immature and transitional B cells postreconstitution in NHP[12] and allowed prolonged graft survival. Mindful of the increased risk of early acute rejection seen in kidney transplant recipients on calcineurin inhibitor-free maintenance regimens after receiving induction with alemtuzumab, a monoclonal antibody to CD52 that depletes both T and B cells,[13-15] we added tacrolimus to the maintenance regimen for the first 6 months. This clinical trial represents the first attempt to actively engender clinical transplantation tolerance by inducing B cell-mediated immune regulation in vivo.

2. Materials and methods

The Immune Tolerance Network trial ITN039ST, Research Study of ATG and Rituximab in Renal Transplantation (RESTARRT; NCT01318915), was a prospective single-arm, open-label study designed to assess the safety and efficacy of rituximab and ATG to facilitate successful IS withdrawal (ISW) in live donor kidney transplant recipients. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review boards of all participating centers.

2.1. Enrollment and study population

Subjects enrolled at 6 kidney transplant centers in the United States from July 2011 until October 2015. Adult, human leukocyte antigen (HLA) unsensitized, Epstein-Barr virus (EBV) seropositive recipients of a primary, $\geq$ 1-haplotype-matched, living related donor kidney allograft were eligible. Those with a history of selected infections (human immune deficiency virus, hepatitis B, hepatitis C, or tuberculosis), malignancy, uncontrolled hyperlipidemia, thrombocytopenia, or glomerular disease with a high risk of recurrence (such as focal segmental glomerulosclerosis) were excluded from participation. All subjects provided written, informed consent. The target accrual was 12 subjects.

2.2. Transplantation and IS

Transplantation was performed using standard surgical techniques followed by standard perioperative management, excepting the immunosuppressive strategy described below. All subjects received chemoprophylaxis against cytomegalovirus

(CMV; 6 months) and *Pneumocystis jirovecii* (12 months) as per the study protocol. Other prophylaxis was given as per the sites' standard of care.

Immune therapy (Fig. 1)[16] began 6 days preoperatively with the first dose of rituximab (375 mg/m^2). The second dose of rituximab was administered on postoperative day one. Premedication for rituximab consisted of diphenhydramine, acetaminophen, and hydrocortisone (100 mg, intravenous [IV]). The first dose of ATG (1.5 mg/kg IV) was given intraoperatively (day 0) following methylprednisolone (250 mg IV). Three additional doses of ATG (1.5 mg/kg each) were given during the first week, preceded by steroids (IV methylprednisolone 100 mg prior to dose 2, IV methylprednisolone or oral prednisone 0.5 mg/kg prior to doses 3 and 4); no steroid therapy was given after the first week. Oral tacrolimus was started on day 0 with a target 12-hour trough level of 6 to 10 ng/mL. Oral sirolimus was started on day 10 posttransplant at 2 mg daily with a target trough level of 8–12 ng/mL. Beginning with subject 2, mycophenolate mofetil (MMF) was administered from days 0 to 12, in order to provide additional IS immediately posttransplant before tacrolimus levels were therapeutic and before sirolimus had been started. In subjects with sirolimus intolerance or tacrolimus toxicity, MMF could be used to supplement or replace either drug.

2.3. Immunosuppression withdrawal

ISW was attempted only in subjects with stable renal function with an estimated glomerular filtration rate (GFR) $\geq$ 50 mL/min/1.73 m^2, no episodes of rejection, and no donor-specific HLA antibody (donor-specific HLA antibody [DSA]). Tacrolimus weaning was initiated between weeks 26 and 38 and completed over 4 to 8 weeks. Sirolimus weaning was initiated between weeks 56 and 80 and completed over 12 to 26 weeks. Those who were ineligible to initiate tacrolimus or sirolimus withdrawal continued the study medications and completed all study visits for standard follow-up. Subjects who attempted but failed ISW for any reason, including rejection, were switched to standard IS, as determined using the site, and underwent standard follow-up. Withdrawal was not reattempted.

2.4. Study endpoints

The primary endpoint was the proportion of transplanted participants who remained off IS for at least 52 weeks without evidence of rejection, based on laboratory data and a protocol biopsy. Secondary endpoints included the incidence of death and graft loss, the incidence and severity of acute rejection, the incidence of adverse events (AEs), and renal function. Given a pilot, exploratory study of 12 subjects, the analyses were to be solely descriptive.

2.5. Clinical and laboratory monitoring

Follow-up visits and monitoring for renal allograft function and blood counts were conducted at least once weekly for the first 8 weeks, then every 2 weeks until week 24 posttransplant. The GFR for analysis was estimated using the 2021 Chronic Kidney Disease Epidemiology Collaboration equation.[16] Subjects who initiated ISW were followed weekly for the first 26 weeks, then every 4 weeks. Subjects who successfully withdrew all immunosuppressive medications underwent 24 weeks of high intensity follow-up (every 2 weeks for the first 8 weeks and every 4 weeks until week 24), followed by 104 weeks of standard follow-up (visits every 12 weeks). Graft dysfunction was defined as a sustained increase in serum creatinine (SCr) level ($\geq$ 25% prior to and during sirolimus withdrawal and $\geq$ 15% after sirolimus withdrawal) compared with baseline.

Pretransplant donor and recipient molecular HLA typing (ranging from low to high resolution) was conducted by each site prior to enrollment. Serum was collected 4 to 8 weeks prior to ISW, every 4 to 8 weeks during ISW and 24 weeks after, and every 4 to 12 weeks thereafter, as well as with every episode of suspected rejection. Serum was screened for class I and II HLA antibodies in real-time by a central laboratory (Emory University, Atlanta, GA) using Flow PRA screening beads (One Lambda, Inc). Screen-positive samples were analyzed using LabScreen Single Antigen Beads (One Lambda, Inc) on the Luminex platform. HLA antibody was considered positive if the mean fluorescence intensity (MFI) was $>$ 1500 and consistent with a known HLA reactivity or pattern.

BK virus levels were monitored using a polymerase chain reaction (PCR) assay at 4, 12, 24, and 52 weeks. Plasma EBV and CMV PCR were monitored every 3 months.

2.6. Kidney biopsies and treatment of acute rejection

Surveillance biopsies were performed prior to the initiation of sirolimus withdrawal, at 2 weeks and 24 weeks during intensive follow-up postwithdrawal, and at approximately 1, 1.5, and 2.5 years after the completion of sirolimus withdrawal. For-cause biopsies were obtained as needed. All biopsies were performed percutaneously under ultrasound guidance with a 15- to 18-gauge needle. Biopsies were read by central and local pathologists and scored according to the Banff 2007 classification.[17] Subjects with biopsy proven acute rejection received treatment for rejection and then resumed/restarted maintenance IS, as per the local institutional standard.

2.7. Mechanistic analyses

Real-time flow cytometry (Roswell Park Cancer Institute, Buffalo, NY) was performed on peripheral blood mononuclear cells (PBMC) collected prior to the first dose of rituximab, day 0, day 1, postoperative weeks 1, 2, 4, 8, 16, and 24, and at multiple time points during the period ISW and through 24 weeks following complete ISW. The data were reviewed for technical and biologic consistency. Additional batch analyses (UCLA, Los Angeles, CA) were conducted on frozen PBMC. Antibodies used for real-time and batched analyses are detailed in Supplementary Tables S1 and S2.

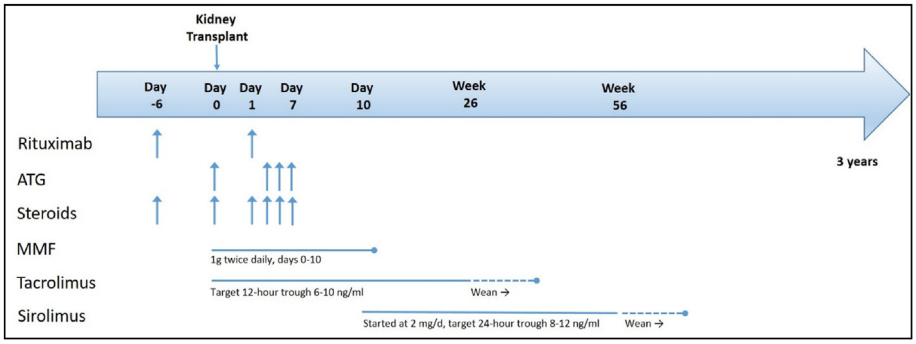

Figure 1. Study design and scheme of immune therapy and immunosuppression withdrawal. ATG, antithymocyte globulin; MMF, mycophenolate mofetil.

3. Results

3.1. Study enrollment and baseline characteristics

Fifteen subjects underwent screening; of these, 10 were enrolled and underwent transplantation (Fig. 2). Demographic and transplant characteristics of the enrolled subjects are listed in Table 1. The calculated panel reactive antibody was 0% in all subjects. Four subjects were one-haplotype matched with their live donor, and the remaining were HLA-identical by descent. Five subjects underwent transplantation preemptively.

3.2. Immunosuppression maintenance

Three subjects were ineligible for ISW due to early acute antibody-mediated rejection (AMR), adverse reaction to study therapy and recurrent immunoglobulin (Ig) A (IgA) nephropathy.

Subject 1 (the first treated subject in the study) had an uneventful postoperative course initially. At 13 days posttransplant, he was diagnosed with C4d+ acute AMR and borderline change and found to have circulating class II DSA. This rejection episode was treated with plasmapheresis, IV steroids, and ATG; sirolimus was discontinued and MMF was started. Graft function improved with treatment. This subject was subsequently maintained on tacrolimus and MMF. At 65 days posttransplant, he had no detectable DSA in circulation, and a follow-up biopsy showed resolution of AMR. After this subject's course was observed, the protocol was modified so that the 2 rituximab doses were administered on preoperative day 6 and on day zero, rather than on days 0 and postoperative day 5.

Subject 8 discontinued study therapy, both rituximab and ATG, on postoperative day 1 due to AEs (described under safety results). This subject moved to safety follow-up and did not receive any further protocol-directed interventions.

Subject 10 was maintained on tacrolimus and sirolimus after day 12, as per study protocol. On day 177 posttransplant, a kidney biopsy performed for graft dysfunction (SCr level 2.3 mg/dL) revealed IgA nephropathy, likely representing a recurrence of her primary disease, and no evidence of acute rejection. The subject was treated with steroids for recurrent glomerulonephritis, sirolimus was stopped, and MMF was restarted. Graft function remained stable, and follow-up biopsies on days 225 and 460 continued to show IgA nephropathy without rejection.

3.3. Immunosuppression withdrawal

Seven subjects initiated ISW, and 6 completed it. Subject 7 had successfully discontinued tacrolimus by postoperative day 231. Despite stable graft function, he was found to have acute T cell-mediated rejection (TCMR) grade 1A on the study protocol biopsy done on postoperative day 430 and was therefore ineligible to initiate sirolimus withdrawal. Given stable graft function, tacrolimus was not reintroduced, and he remained on sirolimus alone. Follow-up study biopsies on postoperative days 462 and 872 continued to show acute TCMR (grades 1A and 1B, respectively), although graft function was stable, and therefore the subject did not receive any therapy for rejection. Approximately 10 months later, the subject developed graft dysfunction and underwent a kidney biopsy on postoperative day 1139, which showed borderline change. At this time, he was treated with steroids with improvement in his graft function, and prednisone was added to sirolimus as part of his maintenance immunosuppressive regimen.

Outcomes of subjects who successfully completed ISW, including the biopsy results, are listed in Table 2. Of these 6 subjects, 4 resumed IS after IS-free periods ranging from 14 to 639 days for biopsies showing acute rejection (n = 3) or recurrent disease (n =1), one subject was lost to follow-up after an IS-free period of 296 days, and one subject remains IS-free for over 9 years with stable SCr levels and no DSA.

3.4. Acute rejection and graft function

Of the 9 subjects in the study who received protocol-directed IS, 1 developed an early acute AMR, 1 developed subclinical TCMR during ISW, and 4 developed TCMR 12 to 452 days following ISW completion. Table 3 lists the characteristics of subjects with acute rejection in the study. All episodes of acute rejection recovered with treatment, and there was a return of graft function to near-baseline values. Figure 3 displays graft function over time in all subjects. The results of all for-cause biopsies conducted during the study are listed in Supplementary Table S3.

3.5. DSA development in the study

As noted previously, only HLA-unsensitized recipients were enrolled in the study. Subject 1 was diagnosed with acute AMR at

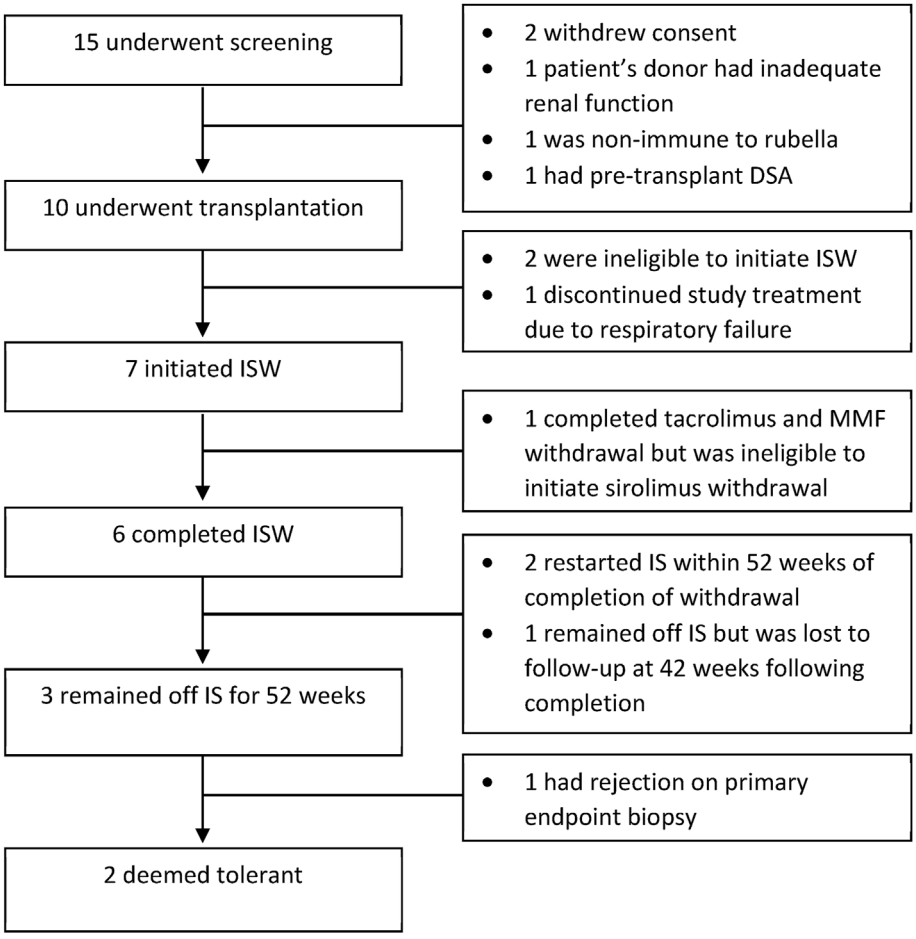

Figure 2. Subject disposition in RESTARRT. *1 subject had acute rejection and restarted IS 162 weeks after being deemed tolerant at the primary endpoint. ATG, antithymocyte globulin; DSA, donor-specific HLA antibody; IS. Immunosuppression; ISW, immunosuppression withdrawal; MMF, mycophenolate mofetil; RESTARRT, Research Study of ATG and Rituximab in Renal Transplantation.

13 days posttransplant and found to have circulating de novo DSA to HLA DR13 (MFI 18000) and HLA DR52 (MFI 11000). DSA declined with treatment and had resolved by 64 days posttransplant. No other subject developed de novo DSA during the study.

3.6. Safety

A total of 72 AEs were reported during the study, of which 15 were serious AEs (SAEs). The most frequently reported nonserious AEs were leukopenia (n = 6) and a decreased lymphocyte

Table 1
Baseline subject characteristics.

ID	Age (y)	Sex	Race	Cause of ESRD	Haplotype-match with donor
1	26	M	White	Obstructive/reflux nephropathy	1
2	63	M	White	IgA nephropathy	2
3	55	M	Pacific Islander	Diabetes mellitus	2
4	29	F	Black	Fibrillary glomerulonephritis	2
5	47	M	White	Glomerulonephritis	2
6	29	M	White	Chemical exposure	2
7	25	M	White	Solitary kidney and hypertension	1
8	34	M	White	Obstructive/reflux nephropathy	1
9	34	M	White	IgA nephropathy	2
10	24	F	White	IgA nephropathy	1

ESRD, end-stage renal disease; Ig, immunoglobulin.

Table 2
Outcomes of subjects who completed ISW.

ID	Completion of ISW (wk posttransplant)	IS-free duration (wk)	Study surveillance biopsies post-ISW (wk post-ISW)[a]					Comments
			≤2 wk	26 wk	52 wk (primary endpoint)	78 wk	130 wk	
2	93	488[b]	Borderline change	No acute rejection	Borderline change	Borderline change	Borderline change	Remains free of immune suppression
3	97	28	No acute rejection[c]	Borderline change	Borderline change	-	-	Resumed immune suppression 126 wk posttransplant (prior to primary endpoint) for-cause biopsy showing ACR IA
4	89	91	Borderline change	Subject declined biopsy	Acute TCMR Banff 1A	-	-	Resumed immune suppression 181 wk posttransplant (postprimary endpoint) for recurrent primary disease. Of note, IS had not been resumed for protocol biopsy 37 wk prior and showed ACR 1A since graft function was stable
5	97	64	No acute rejection	Borderline change	No acute rejection	-	-	Resumed immune suppression 162 wk posttransplant (postprimary endpoint) for-cause biopsy showing ACR 1B
6	87	42	No acute rejection	Subject declined biopsy	-	-	-	Lost to follow-up at 130 wk posttransplant (prior to primary endpoint)
9	80	2	Acute TCMR Banff 1B	-	-	-	-	Resumed immune suppression 82 wk posttransplant (prior to primary endpoint) for protocol biopsy showing ACR 1B

ACR, acute cellular rejection; ISW, immunosuppression withdrawal; TCMR, T cell-mediated rejection.

[a] Biopsy read according to the central pathologist.

[b] At completion of the study, the subject had been free of immune suppression for 130 weeks and continues to remain free of immune suppression.

[c] Central pathology read not obtained, local pathology read presented.

Table 3
Acute rejection grade ≥ Banff IA in the study.

ID	Type and severity of acute rejection	Phase of study	Time posttransplant (wk)	Treatment	Baseline SCr (mg/dL)	SCr at the time of rejection (mg/dL)	Nadir SCr prior to the rejection (mg/dL)	SCr at study completion (mg/dL)	UPCR at study completion
1	Clinical acute AMR	On immune suppression	1	Plasmapheresis, steroids, ATG, IVIg	1.74	2.3	1.8	1.35	0.16
7	Subclinical ACR 1A	During ISW	61	No specific treatment (treated for biopsy showing borderline changes at 162 wk posttransplant)	1.64	1.46	1.24	1.33	0.10[a]
3	Clinical ACR 1A	Post-ISW	126	Steroids, resumption of immune suppression	1.82	1.35	1.18	1.31	0.15[a]
4	Subclinical ACR 1A	Post-ISW	143	No specific treatment. Resumption of immune suppression 37 wk later	0.96	0.88	0.75	0.76	2.86[b]
5	Clinical ACR 1B	Post-ISW	162	Steroids, ATG, and resumption of immune suppression	2.00	2.41	1.39	2.52	0.22[a]
9	Clinical ACR 1B	Post-ISW	82	Steroids and resumption of immune suppression	1.33	1.17	1.11	1.24	0.03

ACR, acute cellular rejection; AMR, antibody-mediated rejection; ATG, antithymocyte globulin; ISW, immunosuppression withdrawal; IVIg, intravenous immunoglobulin; SCr, serum creatinine; UPCR, urine protein creatinine ratio.

[a] UPCR not available at study completion. Last available UPCR presented.

[b] Proteinuria attributed to recurrence of original disease (fibrillary glomerulonephritis).

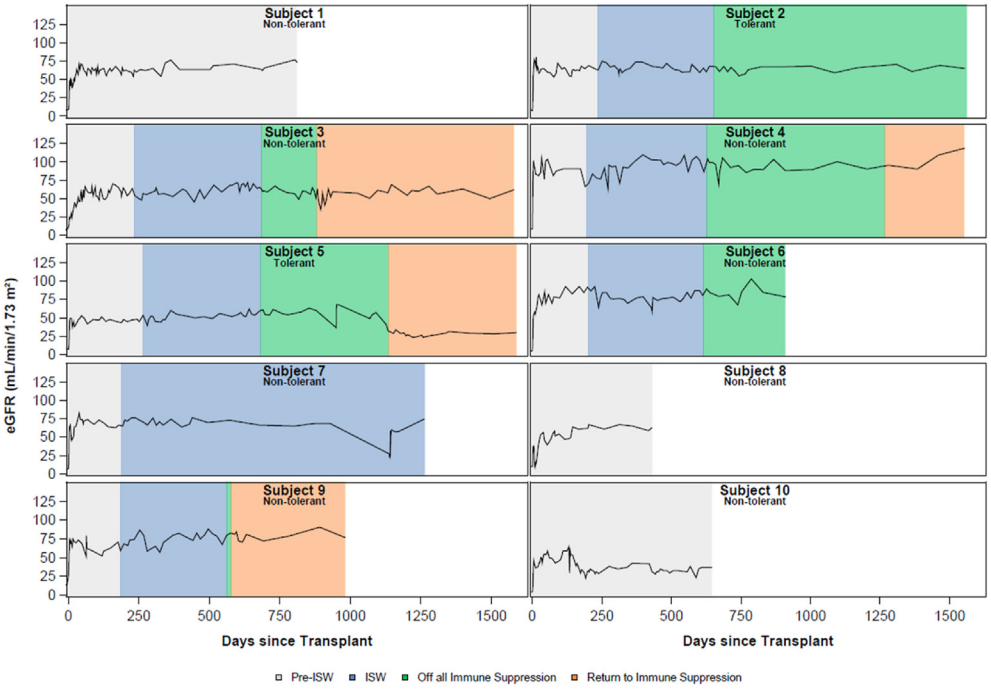

Figure 3. Trajectory of renal allograft function over time for all subjects. The glomerular filtration rate was estimated using the 2021 Chronic Kidney Disease Epidemiology Collaboration equation.[16] Shading indicates the phase of the study during which the measurement was observed. Gray represents the study phase prior to immunosuppression (IS) withdrawal (ISW), blue during ISW, green when free of IS, and orange when immune suppression was restarted after being free of immune suppression. Note that subject 7 initiated ISW but was not able to withdraw fully and remained on sirolimus monotherapy. Tolerance status (at the time of the primary endpoint assessment) is indicated under the subject identifier. Note that subject 5 had acute rejection and restarted IS 162 weeks after being deemed tolerant at the primary endpoint.

count (n = 5). The most frequent SAE was acute rejection (n = 4). Other SAEs included acute respiratory failure (n = 1), deep vein thrombosis (n = 1), incisional hernia (n = 2), seroma (n = 1), tacrolimus and sirolimus nephrotoxicity (n = 1), stroke (n = 1), pyelonephritis (n = 1), nephrolithiasis (n = 1), abdominal hernia (n = 1), and renal injury (n = 1). As described above, 1 subject (subject 8) experienced an SAE that led to the discontinuation of the study drugs rituximab and ATG. This subject had received the first infusion of rituximab 7 days prior to the transplant and the first dose of ATG on the day of the transplant without any issues. On postoperative day one, he developed respiratory symptoms starting one hour after his second dose of rituximab was initiated, which then progressed to acute respiratory distress syndrome, for which he required mechanical ventilation. Imaging studies showed consolidation in both lungs and no evidence of pulmonary embolism. The findings were attributed to pneumonitis, likely caused by rituximab. Both ATG and rituximab were discontinued, and further immunosuppressive medications were prescribed according to the standard of care. He was successfully extubated on postoperative day 9.

One subject developed BK viremia at 83 days posttransplant (peak viral load 47 000 copies/mL) without graft dysfunction, which resolved by day 161 posttransplant. Another subject developed transient CMV viremia at >1 year posttransplant. There were no cases of EBV viremia or posttransplant lymphoproliferative disorder. There were no cases of death or graft loss in the study.

The study met a stopping rule when a fourth subject (out of 10 subjects who had received a transplant) experienced acute rejection. Further enrollment was halted after review by the National Institute of Allergy and Infectious Diseases Transplant Data and Safety Monitoring Board. Previously enrolled subjects were permitted to continue with the study protocol, including ISW. After a fifth subject experienced acute rejection, the Data and Safety Monitoring Board recommended discontinuation of protocol-directed IS management in all subjects. The protocol was amended for safety follow-up only, and immunosuppressive medications were prescribed by the subjects' treating physicians according to the site's standard of care. As noted previously, all episodes of acute rejection recovered clinically.

3.7. Lymphocyte repopulation

Real-time flow cytometry was performed on fresh PBMC to characterize and quantify the reconstitution of natural killer (NK), B, and T cells posttransplant. Figure 4 depicts the average and absolute number of cells detected in subjects at the indicated study weeks. $CD3^{-}CD56^{+}$ NK cells began to repopulate the periphery by 2 weeks posttransplant, with repopulation composed of primarily mature $CD56^{lo}$ NK cells (Fig. 4A). $CD19^{+}$ B cell numbers began to rebound around 26 weeks posttransplant (Fig. 4B). $IgD^{+}CD27^{-}$-naïve B cells dominated this repopulation and on average returned to predepletion numbers as subjects began ISW. Changes in B and T cell populations per subject and across study subjects are illustrated in Supplementary Figures S1 to S5. The numbers of both total $CD19^{+}$ B cells and $IgM^{+}IgD^{+}$ transitional B cells correlated inversely with the concentration of B cell activating factor detected in subjects' serum (Supplementary Fig. S6).

Repopulation in the T cell compartment was dominated by memory cells, in contrast to the pretransplant and predepletion populations, in which the majority of T cells were naïve ($CD45RA^{+}CD197^{+}$). Total $CD8^{+}$ T cells began to rebound by 2 weeks posttransplant, and repopulation was dominated by $CD45RO^{+}CD197^{-}$ T effector memory (TEM) and CD45RA+$CD197^{-}$ T effector memory RA^{+} cells (TEMRA)

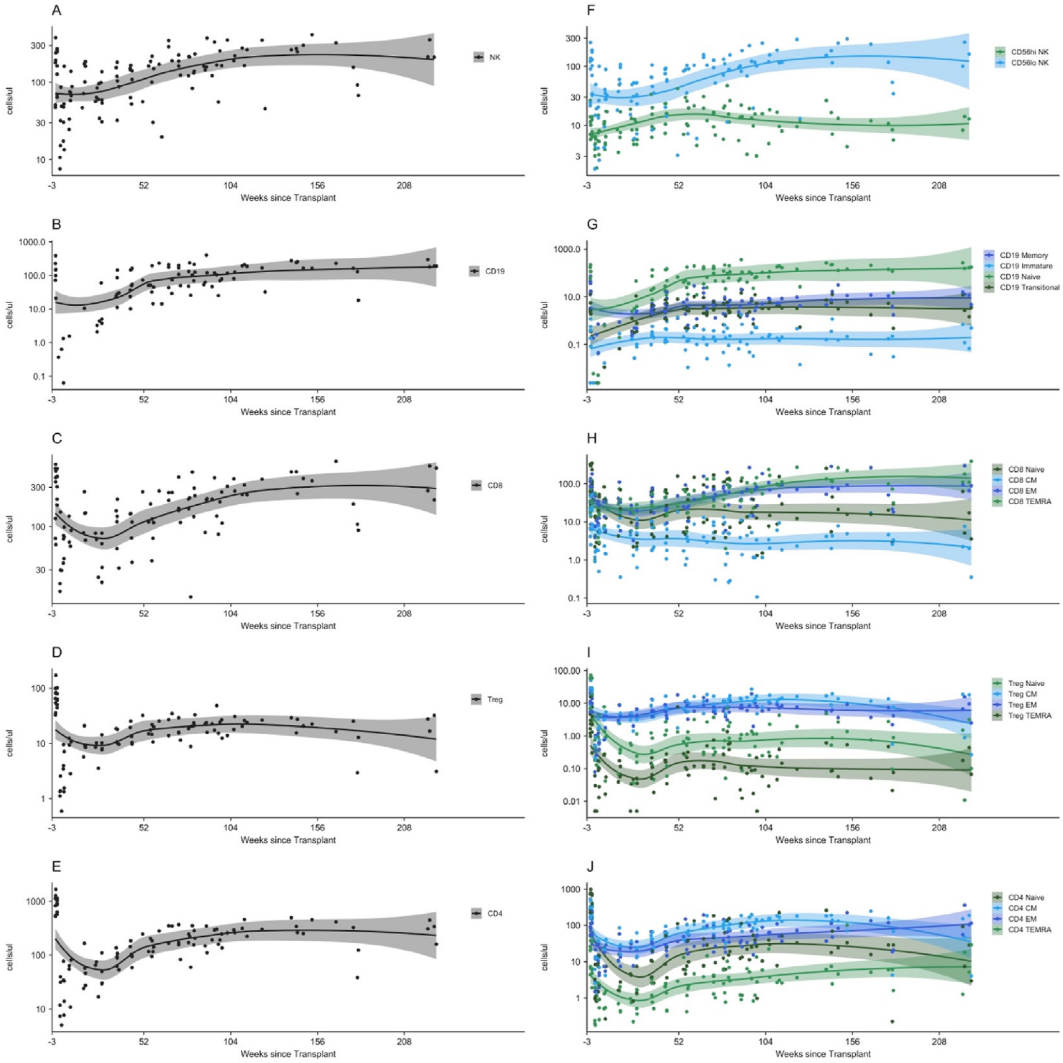

Figure 4. Lymphocyte reconstitution of the periphery following peritransplant T and B cell depletion. Lines represent the average number of lymphocytes (cells/uL) detected using real-time flow cytometry performed on fresh peripheral blood mononuclear cell from subjects who received updated study treatment and protocol-directed intervention (all subjects excluding 1 and 8). Shaded areas represent the 95% confidence interval. Dots represent the number of lymphocytes in the indicated population per subject. Black lines/dots indicate the total number of natural killer (NK) cells (A), CD19+ B cells (B), CD8+ T cells (C), regulatory T cells (Tregs) (D), or CD4+ T cells (E), as indicated. Colored lines/dots demonstrate subpopulations of NK cells (F), CD19+ B cells (G), CD8+ T cells (H), Treg (I), or CD4+ T cells (J).

(Fig. 4C). $CD4^+CD127^-CD25^{hi}$ regulatory T cells (Treg) began to repopulate next, with noticeable increases in numbers 4 to 8 weeks posttransplant. TEM and $CD45RO^+CD197^+$ T central memory (TCM) cells dominated the repopulation of Tregs (Fig. 4D). Lastly, total $CD4^+$ T cell numbers began to rebound after week 26 posttransplant. Similar to the Treg compartment, total $CD4^+$ T cell repopulation consisted mostly of TEM and TCM (Fig. 4E). These findings suggest that T and B cells repopulate the periphery with different kinetics and to a different extent following cell depletion, transplant, and IS.

Batched flow cytometry was also performed on frozen PBMC from selected time points to further characterize the repopulating T cells. Figure 5 depicts the percentage of $CD3^+$ lymphocytes that are $CD4^+$ (Fig. 5A) or $CD8^+$ (Fig. 5E). The 3 oldest subjects in the study, aged 63, 55, or 47 years old at the time of transplant, had an increased proportion of $CD4^+$ (Fig. 5B-D) and $CD8^+$ (Fig. 5F-H) T cells that were $CD57^+$, $Eomes^+$, or $TIGIT^+KLRG1^+$ at the end of study compared with younger subjects, aged 24 to 34 years old at the time of transplant. Of note, 2 out of 3 of the oldest subjects met the study definition of operational tolerance. This observation might suggest that subject age or T cell exhaustion affects the phenotype of repopulating T cells.

4. Discussion

This study represents the first effort to induce transplant tolerance in kidney transplant recipients using thymoglobulin and rituximab. Unfortunately, of the 10 patients who underwent a

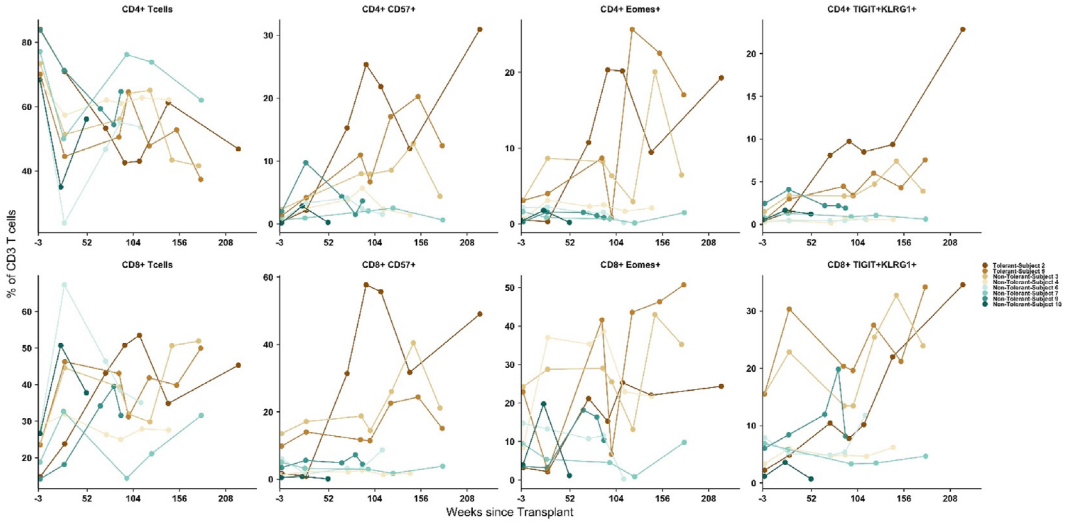

Figure 5. CD57+, Eomes+, and TIGIT+KLRG1+ CD4+ and CD8+ T cells accumulate in older subjects during T and B cell repopulation of the periphery. Batched flow cytometry was performed on frozen peripheral blood mononuclear cells at the conclusion of the trial. CD4+ (A-D) and CD8+ (E-H) T cells were analyzed. Data are represented as the percentage of CD3+ lymphocytes (A and E) or the percentage of CD3+CD4+Foxp3- (B-D) or CD3+CD8+ (F-H) lymphocytes. Each colored line represents a single subject who received the modified treatment and protocol-directed intervention (all subjects, excluding 1 and 8). Green and tan-hued lines represent subjects that did not meet the protocol definition of tolerance. The lightest green line represents the subject who completed immunosuppression (IS) withdrawal (ISW) and was lost to follow-up before primary endpoint adjudication. The brown-hued lines represent subjects that met the protocol definition of tolerance at the time of the primary endpoint assessment. Note that subject 5 had acute rejection and restarted IS 162 weeks after being deemed tolerant at the primary endpoint. The 3 subjects in brown and dark tan were 63, 55, or 47 years old at the time of transplant. The other subjects were 24 to 34 years old at the time of transplant.

transplant in the study, only 6 completed ISW, and 2 met the primary endpoint. We conclude that this protocol was insufficient to induce tolerance in the majority of participants.

These results differ from prior translational work in NHP, in which the combination of T and B cell depletion with transient sirolimus maintenance resulted in prolonged islet allograft survival.[12] In these animals, a shift toward nonisotype-switched immature or transitional B cells occurred during lymphocyte repopulation, and the ratio of immature and transitional B cells to mature, isotype-switched B cells correlated with tolerance. Although all patients in our study demonstrated peripheral depletion of B cells, we did not detect any significant degree of expansion of immature-stage transitional B cells compared with baseline. This difference in repopulation may account for the lack of success of the clinical protocol compared with preclinical experience and is consistent with the finding that the frequency of relapse in rituximab-treated patients with autoimmune disease is correlated with the phenotypic constitution of the repopulating B cells.[18]

Another possibility is that the degree of depletion of B cells achieved by 2 doses of rituximab was inadequate. In patients with lupus nephritis treated with 4 doses of rituximab, the achievement of complete peripheral depletion (defined as 0 cells/μL), was strongly associated with clinical response.[19] Complete peripheral depletion was seen in 3 patients in our study; none experienced acute rejection. One remains IS-free for over 9 years, the second completed ISW and was lost to follow-up after their last study visit at 296 days post-ISW (911 days posttransplant) prior to the evaluation for the primary endpoint, and the third patient restarted IS due to a recurrence of IgA nephropathy in the allograft.

Further, the effect of rituximab on the immune response may depend not merely on the extent of peripheral B cell depletion but also on the functional properties of the remaining B cells.[20] B cells can persist in protected microenvironments, as demonstrated in the lymph nodes of patients treated with rituximab prior to kidney transplantation.[21,22] Another potential explanation is that large increases in B cell activating factor, occurring secondary to peripheral B cell depletion, may in turn promote the survival of nondepleted B cell populations and contribute to rituximab's lack of efficacy.[23] Finally, we observed a sustained decrease in the proportion of $CD19^{+}CD24^{hi}CD27^{+}$ B cells, which conflicts with reports of a preferential increase in regulatory B cells with rituximab treatment.[24,25] The difference in results observed in our study may be due to the difficulty of identifying regulatory B cell populations and the effect of factors such as dose and timing of rituximab therapy, as well as the immunologic environment, in determining the effect on specific B cell subpopulations.

Of the 6 patients with acute rejection in the study, only 1—the first patient treated in the study—had AMR, occurring on day 13 posttransplant. This patient received rituximab only postoperatively (days 1 and 9), and did not start an antiproliferative agent until day 10. The study regimen was modified in subsequent patients such that rituximab was initiated 6 days preoperatively, and MMF added for the first 12 days. No additional cases of AMR were seen in the study and no other patient developed DSA during the study.

Of note, a 6-fold higher rate (83% vs 14%) of acute TCMR was observed in kidney transplant recipients receiving rituximab as induction therapy in combination with early steroid withdrawal,[26] causing that trial to be stopped early. When we designed our study, we felt that the addition of thymoglobulin to rituximab would mitigate the increased risk of cell-mediated rejection observed with rituximab alone. Higher rates of early acute rejection in kidney transplant recipients, both cellular and humoral, have also been seen with alemtuzumab, which depletes both T and B cells, especially when followed by calcineurin inhibitor-free regimens.[13-15] In our study, no cases of acute TCMR occurred prior to initiating ISW while patients were on tacrolimus and sirolimus. Nevertheless, the observed changes in the T cell compartment could underlie the cases of acute TCMR that occurred in 5 patients >1 year posttransplant, during or after ISW. $CD8^+$ T cells switched from a mostly naïve phenotype to a TEMRA/TEM phenotype. $CD4^+$ and $CD4^+$ Treg T cell numbers did not recover to preinduction levels by the end of the study. Additionally, in contrast to being composed of mostly naïve ($CD4^+$ T cells) or a combination of naïve and TCM (Tregs) as they were prior to induction therapy, $CD4^+$ T cell subsets at the end of the study were made up of mostly TEM and TCM. A similar postreconstitution T cell profile has been seen following alemtuzumab or thymoglobulin (without rituximab).[27]

With respect to safety, although only a minority of patients enrolled reached the primary endpoint of rejection-free graft survival at 52 weeks, there were no deaths or graft losses. All patients with acute rejection had a good response to treatment, with a recovery of renal function close to their baseline prior to the rejection episode. There were no cases of serious opportunistic infections or malignancies. Although tolerance was not routinely achieved in this study, half of the study (5 of 10) participants succeeded in weaning and achieving an IS-free period of at least 6 months.

Overall, the results of this study, along with the observations from previous studies incorporating rituximab and alemtuzumab as induction therapy, suggest that the impact of B cell depletion on the overall alloimmune response is complex and variable and not necessarily protolerogenic. A potential target for protocol refinement is reducing the amount of B lymphocyte stimulator (BLyS) available during immune reconstitution. As well-described by others and documented herein, B cell depletion induces a reciprocal rise in BLyS levels that lessens the stringency of B cell negative selection. Neutralizing BLyS may better promote the generation of tolerogenic cells. Ultimately, a better understanding of the mechanisms of repopulation of B cells and T cells following depletion and factors modulating the influence of anti-CD20 antibodies on these responses is required for the design of more selective and effective regimens for tolerance induction in transplantation.

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Data availability

The data that support the findings of this study are openly available in Trial Share at https://www.itntrialshare.org. Subject IDs in ITN TrialShare corresponding to subject numbers in the manuscript are listed in Supplementary Table S4.

Declaration of competing interest

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2024.03.007.

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