

# B Cell Receptor Genes Associated With Tolerance Identify a Cohort of Immunosuppressed Patients With Improved Renal Allograft Graft Function

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Surprisingly, patients consistently predicted to be tolerant displayed a statistically and clinically significant improvement in estimated glomerular filtration rate that increased over time following transplantation. These findings indicate that the frequency of patients consistently predicted to be tolerant is sufficiently high to be clinically relevant and confirm recent findings by others that immunosuppressive agents impact putative biomarkers of tolerance. The association of a B cell–based “signature” with graft function suggests that B cells may contribute to the function/survival of transplanted kidneys.

**Abbreviations:** CNI, calcineurin inhibitor; CTOT, Clinical Trials in Organ Transplantation; DMSO, dimethyl sulfoxide; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ITN, Immune Tolerance Network; LDA, Linear Discriminant Analysis; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin; non-TOL, not predicted tolerant; PBMC, peripheral blood mononuclear cell; RIN, RNA integrity number; RNA, ribonucleic acid; SRTR, Scientific Registry of Transplant Recipients; TOL, predicted tolerant

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## Introduction

Investigators continue to explore strategies to minimize or avoid the long-term requirement for conventional immunosuppressive agents used in solid organ transplantation due to their toxicities. Reports of renal transplant recipients displaying spontaneous operational tolerance to their allografts (1–4), and more recently reports of approaches that intentionally induce operational tolerance to transplanted kidneys (5–7), suggest that long-term graft survival in the absence of chronic immunosuppression and its associated morbidities may be possible. However, attempts at immunosuppressive drug minimization (8–10) as well as the complete withdrawal of immunosuppression (6,7) have shown that recovery of alloimmune effector mechanisms and graft injury occur in a substantial proportion of individuals. A personalized approach to patient management requires the

**We previously reported that two B cell receptor genes, IGKV1D-13 and IGKV4-1, were associated with tolerance following kidney transplantation. To assess the potential utility of this “signature,” we conducted a prospective, multicenter study to determine the frequency of patients predicted tolerant within a cohort of patients deemed to be candidates for immunosuppressive minimization. At any single time point, 25–30% of patients were predicted to be tolerant, while 13.7% consistently displayed the tolerance “signature” over the 2-year study. We also examined the relationship of the presence of the tolerance “signature” on drug use and graft function. Contrary to expectations, the frequency of predicted tolerance was increased in patients receiving tacrolimus and reduced in those receiving corticosteroids, mycophenolate mofetil, or Thymoglobulin as induction.**

identification and validation of biomarkers that detect patients who can maintain stable renal allograft function with reduced or eliminated immunosuppression.

Several groups have reported that spontaneously tolerant kidney transplant recipients harbor changes in their B cell compartment (1,3,11,12). We have previously reported that spontaneously tolerant kidney transplant recipients are characterized by an increase in both selected B cell–derived transcripts and numbers of naïve and transitional B cells in peripheral blood (1). More recently, we reported that in spontaneously tolerant individuals these findings are stable over time and that similar changes can be observed in kidney transplant recipients in whom tolerance was induced by transient mixed chimerism (13).

Among other factors, the potential utility of our (or any) B cell “signature” is linked to its prevalence within the renal transplant population. An exceedingly rare signature (e.g., 1 in 1000) would have limited clinical utility even if it was highly predictive of tolerance. As a next step in considering how this B cell signature might be applied clinically, we undertook a study jointly sponsored by the Immune Tolerance Network (ITN) and the Clinical Trials in Organ Transplantation (CTOT) consortium (ClinicalTrials.gov Identifier: NCT01516177). The two aims of this study were (i) to determine the prevalence and consistency of the ITN B cell signature, previously identified in spontaneously tolerant kidney transplant recipients, in patients receiving chronic immunosuppression, and (ii) to investigate the impact of specific immunosuppressive agents on the prevalence of this signature.

Here we report that the “signature” is observed in a significant proportion of kidney transplant recipients receiving chronic immunosuppression (25–30% at any given time point) and that a subset of these patients maintain this signature over a 2-year period (13.7% at all three time points). Furthermore, from our data, it appears that the choice of immunosuppressive regimens is associated with the prevalence of this signature. Unexpectedly the prevalence of the signature was increased in individuals receiving tacrolimus and reduced in individuals receiving corticosteroids, mycophenolate mofetil (MMF), and Thymoglobulin induction. While the number of patients was small, the incidence of the tolerance signature was reduced in patients receiving a mammalian target of rapamycin (mTOR) inhibitor. Importantly, individuals who consistently displayed the signature had better renal function compared to those who failed to display the signature. The association of this “signature” with better renal allograft function provides some of the first data in humans that B cells may be associated with protective, as well as harmful, effects on transplanted kidneys and supports further studies aimed at defining the utility of

this signature in guiding decisions about immunosuppressive management.

## Materials and Methods

### Patients

Associating Renal Transplantation with the ITN Signature of Tolerance (ARTIST) was a multicenter observational study of adult renal transplant participants that enrolled a total of 248 patients, most with calcineurin inhibitor (CNI)-based immunosuppression (Table 1). Targeted enrollment criteria were to recruit 250 participants, with at least 25 patients receiving Campath induction therapy and 25 participants receiving mTOR inhibitors (sirolimus or everolimus) at time of enrollment who had not received calcineurin inhibitors at least 30 days prior to enrollment. Peripheral blood and peripheral blood mononuclear cell (PBMC) samples were collected at three time-points 1 year apart to assess prevalence of the tolerance signature at specific time-points, its stability over time, and its association with clinical variables of interest. No sample imputations were performed for missing data due to no sample collection or exclusion due to assay QA as mentioned below.

### Gene expression

The gene expression multiplex assay platform used in developing the tolerance-associated biomarker signature (1) was no longer commercially available; therefore, reverse transcriptase polymerase chain reaction TaqMan assays were performed specifically for IGKV1D-13, IGKV4-1, and the housekeeping/control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The gene primer sets and probes for IGKV1D-13 and IGKV4-1 were as follows:

IGKV1D-13: Forward Primer: GGGCTTCTGCTGCTCTGG  
Reverse Primer: TGGAGACTGGGTCAACTGGAT  
Probe: FAM-CCAGGTGCCAGATGTG-MGBNFQ

IGKV4-1: Forward Primer: GACCCAGGTCTTCATTTCTCTGTT  
Reverse Primer: GAGACTGGGTCATCACGATGTC  
Probe: FAM-TAGGCACCAGAGATCC-MGBNFQ

For GAPDH, assays were performed using reagents from ThermoFisher Scientific (ThermoFisher [Waltham, MA] catalog # 4333764F, Probe Exon Location:3, Amplicon Size: 122, Corresponding TaqMan Assay ID: Hs99999905\_m1). No effective assay primer probes for IGLL1 were found during TaqMan assay validation experiments using previously published ITN-507, termed FACTOR samples. FACTOR reference sample results using only IGKV1D-13 and IGKV4-1 gave consistent results to the predicted tolerant (TOL) and non-TOL classifications from the

**Table 1:** ARTIST samples tested for prevalence of the TOL signature

Drug regimen	Number of participants
Campath & mTOR inhibitor	13
Campath only	36
Other (CNI-based regimen)	176
mTOR inhibitor only	23
Total standard immunotherapy participants	248

CNI, calcineurin inhibitor; mTOR, mammalian target of rapamycin; TOL, tolerant.

previously published assay platform data, demonstrating IGLL1's contribution as minimal using the TaqMan platform compared to the other two genes.

RNA (ribonucleic acid) was purified from whole blood tempus tubes using 5' RNA extraction kits (5 Prime, No. 2302100) and eluted in final volume of 40  $\mu$ L. RNA quality and concentration was determined by NanoDrop. Samples with RNA concentrations less than 0.1  $\mu$ g/ $\mu$ L, and/or RNA integrity number (RIN) scores <6 were excluded from analysis. All ARTIST samples for the three time-points and FACTOR control samples were run in a single batch to minimize systematic artifacts due to processing. Gene expression measures were normalized to GAPDH.

#### Flow cytometric analysis

Batched flow cytometric analysis was performed on approximately 10 million PBMC frozen in 20% dimethyl sulfoxide (DMSO)/human AB serum. The combined memory and transitional B cell (M+T) flow panel comprised the following markers: Live/Dead cells; CD3; CD19; IgD; CD27; CD24; CD38; CD21; CD23; CD95; 9G4; and mitotracker green. Note that mitotracker green is used to identify naïve B cells, as extrusion of this dye is mediated by the ABCB1 transporter, which is not expressed in transitional or memory B cells. This same M+T panel was used in the previously published longitudinal analysis of B cell markers in FACTOR participants (13). Flow cytometry result data was analyzed as both absolute measures (cells/ $\mu$ L) and frequency of lymphocytes and CD19<sup>+</sup> B cells. Absolute measures were extrapolated from fresh complete blood count (CBC) data obtained at the same visit as tested frozen PBMC with the formula:

#CD19<sup>+</sup> B cells per $\mu$ L = [(% of CD19<sup>+</sup> B cells in lymphocyte population from frozen flow)  $\times$  (# of lymphocytes from CBC)]/(100%). As with the TaqMan assay, a subset of 10 tolerance FACTOR participants were processed in parallel to confirm assay comparability to prior results published (1,13).

#### Statistical analysis

A Linear Discriminant Analysis (LDA) model using the formula

$$P_{TOL} = \frac{\exp(\beta_0 + \beta_1 G_A + \beta_2 G_B)}{1 + \exp(\beta_0 + \beta_1 G_A + \beta_2 G_B)}$$

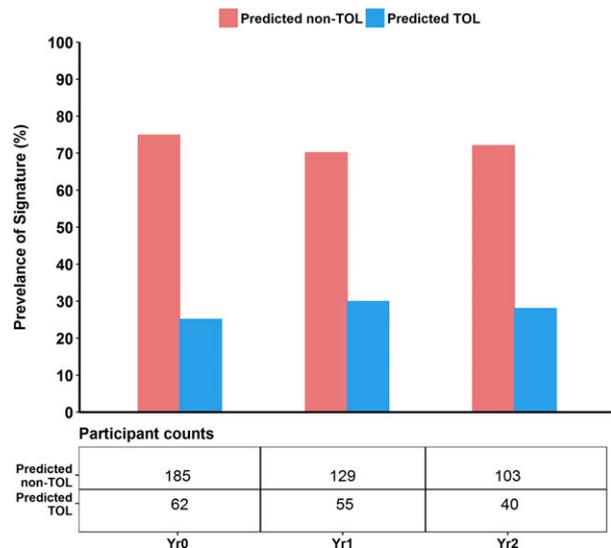
( $\beta_i$  is the coefficient, and  $G_i$  is the expression level for each gene) was trained using FACTOR samples (17 tolerant and 20 on standard immunosuppression) that were run in parallel with ARTIST samples to generate model coefficients using the TaqMan gene expression results. The LDA model and coefficients were then applied to ARTIST participants with gene expression data for testing of the prevalence of the tolerance signature.

For gene expression and flow cytometry analysis, statistical tests were performed using the SAS 9.4 software, using unpaired Student t-tests to compare differences between groups with continuous data within a time point. Whereas differences in B cell counts between different drug regimens were analyzed by one-way analysis of variance, a Fisher exact test was used for comparison of proportions. Significance levels for multiple comparisons were adjusted using Benjamini-Hochberg procedure. A p-value of <0.05 was considered statistically significant for all statistical tests. Normalized gene expression of IGKV1D-13 and IGKV4-1 were calculated by normalizing the gene expression measures by Total B-Cell (CD19<sup>+</sup>) absolute measures. All result data and statistical code associated with figures are available on the ITN TrialShare analysis portal (<https://www.itntrialshare.org/ARTIST.url>).

## Results

### Prevalence and stability of the ITN tolerance signature

In order to determine how frequently the ITN tolerance signature occurred in renal transplant recipients, and whether or not it remained stable over time, we enrolled 248 renal allograft recipients at 8 US transplant centers. All patients were 1–5 years posttransplant, had a calculated glomerular filtration rate (GFR) of  $\geq 45$  mL/min/1.73 m<sup>2</sup>, and had not had a rejection episode for at least 1 year (see Materials and Methods and for further details). Of the 248 patients enrolled, 247 provided samples at study entry, 184 provided samples 1 year later, and 124 provided samples at both 1 and 2 years after study initiation (Figures 1 and S1). At each of the three time points, between 25% and 30% of the patients displayed our previously defined two-gene signature of tolerance (Figure 1—predicted tolerant, denoted as “Predicted TOL” in the figures). While in the majority of patients the tolerance signature was not consistently observed, it was detected at all time points in 17 of the 124 (13.7%) patients for whom all 3 samples were tested. Conversely, 71 patients were consistently predicted to not be tolerant (non-TOL). The quantitative nature of the assay results for those consistently predicted as TOL or non-TOL is shown in Figure S2, which displays the degree to which patients in those groups are above or below the cut-off, respectively. With the exception of the proportion of patients who received a kidney from a living donor, which was higher in the cohorts consistently predicted to be nontolerant, there were no statistically significant differences in any of the demographic or other characteristics between groups (Table 2). These results



**Figure 1: Prevalence of TOL signature over the three time points.** Participants predicted TOL and non-TOL remain relatively stable over time. [https://www.itntrialshare.org/ARTIST\\_fig1.url](https://www.itntrialshare.org/ARTIST_fig1.url) non-TOL, not predicted tolerant; TOL, predicted tolerant.

**Table 2:** Demographic and clinical characteristics: all p-values for comparisons between Consistently Predicted TOL and Consistently Predicted non-TOL >0.05 except as noted below (\*)

	Consistently predicted TOL (n = 17)	Consistently predicted non-TOL (n = 71)	Variable predicted TOL (n = 160)
Race, n			
Asian	1	3	7
Black	4	7	36
White	10	59	100
Other	2	2	14
Data missing, n			3
Ethnicity, n			
Hispanic or Latino	4	7	18
Not Hispanic or Latino	12	58	123
Unknown	1	6	19
Gender, n			
Female	10	28	60
Male	7	43	100
*Donor type, n			
Living-related	3	32	51
Living-unrelated	3	24	34
Deceased	11	15	73
Data missing, n			2
Age at enrollment (yrs, mean [SD])	55 (9.7)	51 (11.2)	49 (10.9)
Age at transplantation (yrs, mean [SD])	52 (9.9)	48 (11)	47 (10.7)
Interval between transplant and enrollment (yrs, mean [SD])	3 (1.4)	3 (1.3)	2 (1.1)
Donor age (yrs, mean [SD])	35 (16.1)	41 (15.2)	38 (14.4)
Documented episodes of acute rejection, n <sup>1</sup>			
Mild acute cellular rejection (Grade IA)		1	3
Mild acute cellular rejection (Grade IB)			1
No acute rejection	12	58	135
Data missing, n	5	12	22
Proteinuria (>30 mg/dL), n			
<30 mg/24 h	14	48	120
≥30 mg/24 h	2	14	21
Data missing, n	1	9	19
Study status, n			
Completed per protocol	17	71	110
Lost to follow-up			18
Death			3
Voluntary withdrawal			8
Other			21
Donor-specific antibodies, (positive participants/total participants tested)			
Class I	0/12	6/59	6/137
Class II	0/12	7/59	15/135
Either Class I or Class II	0/12	10/59	18/137
Data missing, n <sup>2</sup>	5	12	23 & 25
HLA mismatch, (mean [SD])			
Class I	2.47 (1.37)	2.28 (1.26)	2.40 (1.40)
Class II	1.38 (0.62)	1.11 (0.75)	1.08 (0.74)
Data missing, n			1
Panel reactive antibodies (positive participants/total participants tested)			
Class I			
0%	6/12	37/59	86/137
1–≤10%		7/59	15/137
10–≤50%	5/12	13/59	25/137
50–≤100%	1/12	2/59	11/137
Class II			
0%	11/12	49/59	104/137
1–≤10%			5/137

(Continued)

Table 2. Continued

	Consistently predicted TOL (n = 17)	Consistently predicted non-TOL (n = 71)	Variable predicted TOL (n = 160)
10-≤50%	1/12	8/59	13/137
50-≤100%		2/59	15/137
Data missing, n	5	12	23

SD, standard deviation; TOL, tolerant.

<sup>1</sup>One participant in Variable Predicted Group had both Grade IA and IB mild acute cellular rejection episodes.

<sup>2</sup>Twenty-three participants missing data for Class I and 25 participants missing data for Class II in Variable Predicted TOL.

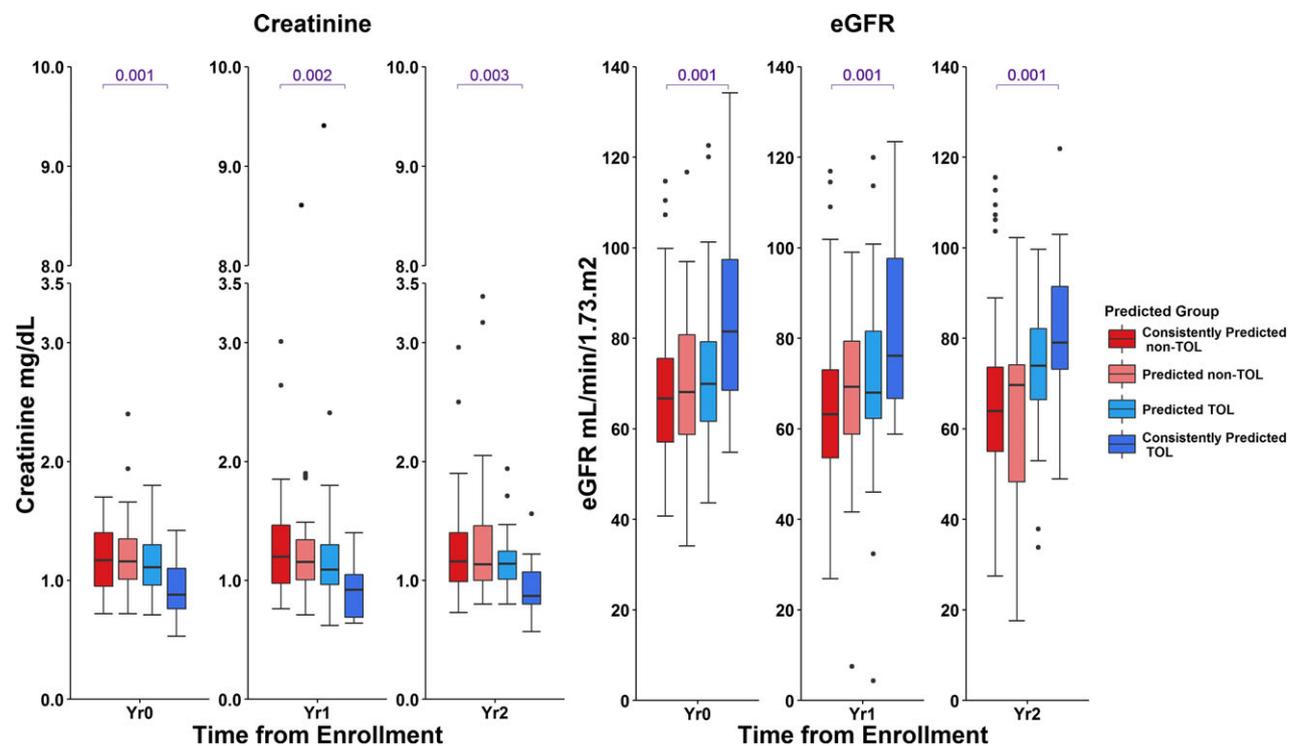
\*p-value <0.05 for comparison between Consistently Predicted TOL and Consistently Predicted non-TOL groups.

demonstrate that our previously described signature of tolerance identified in spontaneously tolerant patients is also consistently detected in a nontrivial number of stable renal allograft recipients receiving immunosuppression.

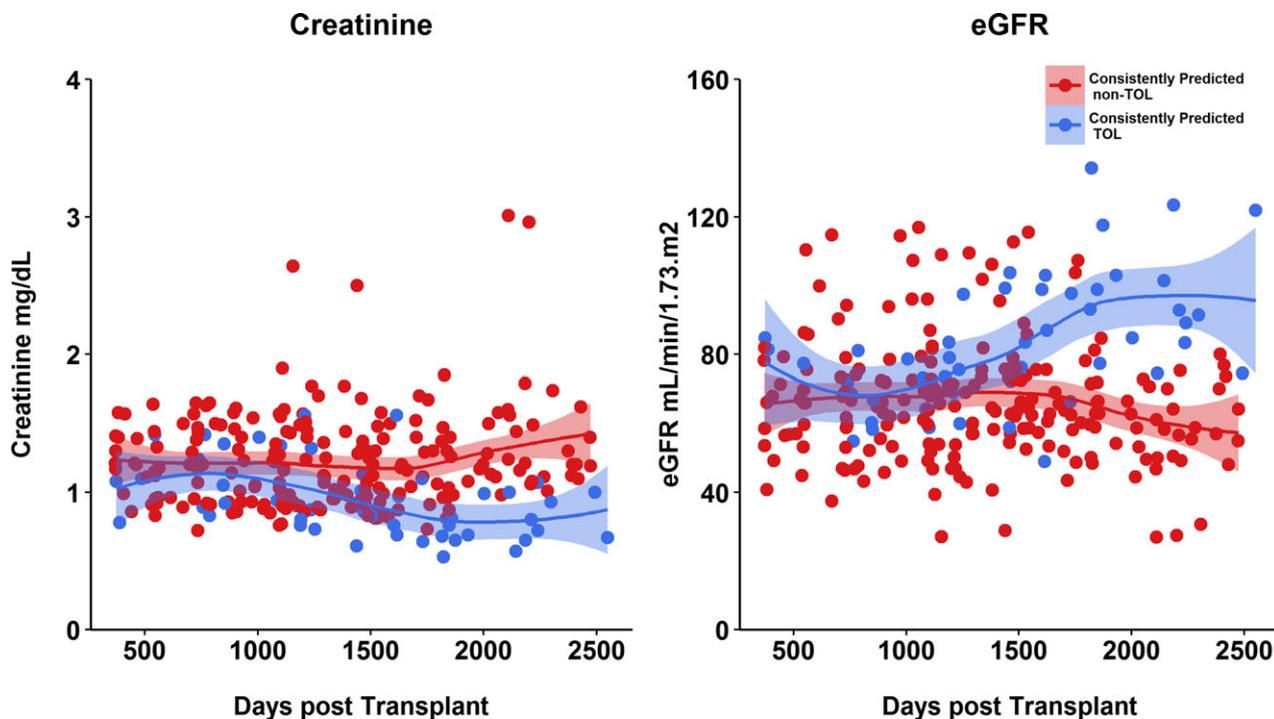
### Relationship of predicted TOL-status to graft function

We first asked if there was a relationship between allograft function and the status of patients (i.e. predicted TOL versus predicted non-TOL). For the initial analysis, we examined creatinine and estimated glomerular

filtration rate (eGFR) in our patients at each of the three study time points based on a TOL versus non-TOL prediction at that time point. As shown in Figure 2, renal function (by either measure) was comparable in patients predicted TOL or non-TOL at any single determination. However, restricting the analysis to the 17 patients who were consistently predicted TOL revealed that these individuals had significantly better renal function (lower serum creatinine, higher eGFRs) than all other groups, particularly those who were consistently predicted to be non-TOL (Figure 2). Moreover, the differences in eGFR remain statistically significant even when correcting for



**Figure 2: Creatinine and eGFR levels between 17 consistently Predicted TOL (dark blue), Predicted TOL at that visit but not for all three time points (light blue), Predicted non-TOL (light red) at that time point, 71 consistently Predicted non-TOL (red).** Participants consistently Predicted TOL have lower creatinine and higher eGFR compared to the other groups. Creatinine and eGFR are statistically significant for 71 consistently Predicted non-TOL versus 17 consistently Predicted TOL. [https://www.intrialshare.org/ARTIST\\_fig2.url](https://www.intrialshare.org/ARTIST_fig2.url). eGFR, estimated glomerular filtration rate; TOL, tolerant.



**Figure 3: Creatinine and eGFR levels between 17 consistently Predicted TOL versus 71 consistently Predicted non-TOL.** Solid line shows smooth line for each group with shading representing 95% confidence intervals. Data shown for all three time points as available per participant. [https://www.itntrialshare.org/ARTIST\\_fig3.url](https://www.itntrialshare.org/ARTIST_fig3.url). eGFR, estimated glomerular filtration rate; TOL, tolerant.

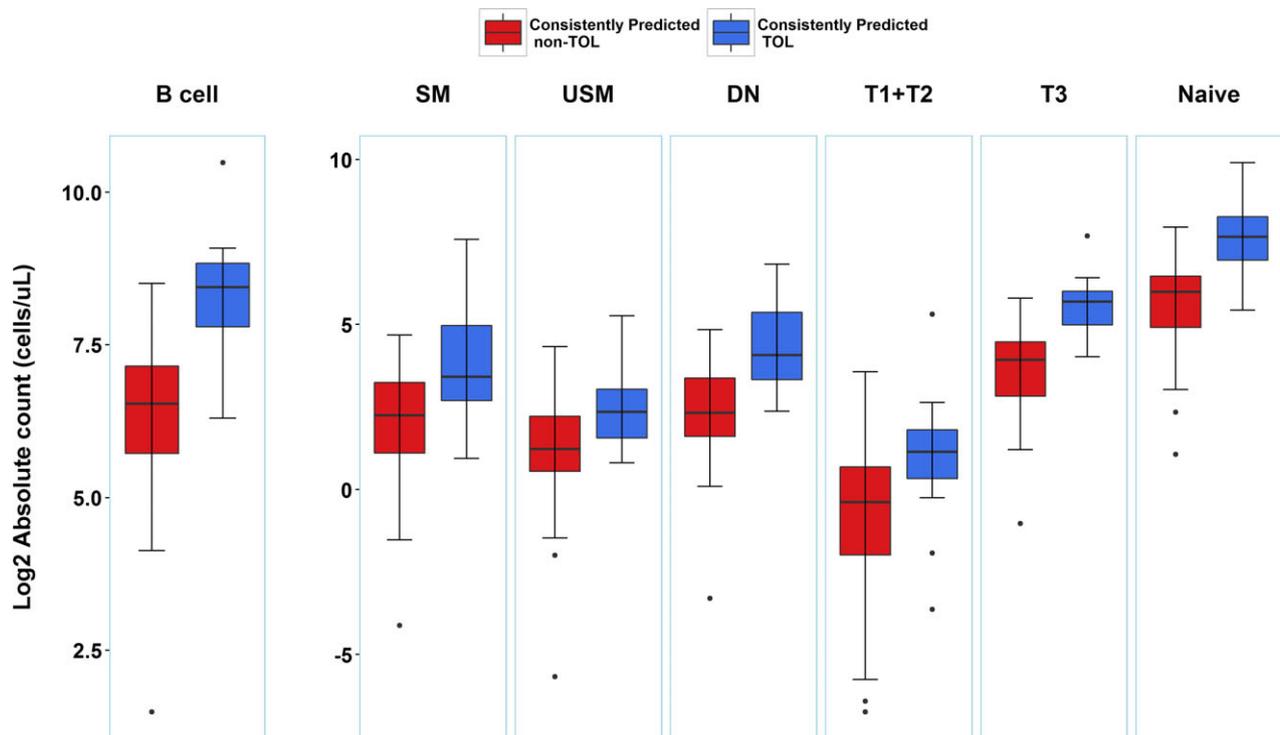
other variables including degree of proteinuria, donor type (living vs. deceased) and type of immunosuppression, and when correcting for multiple testing across the three time points. It is worth noting that these findings are unlikely to be related to differences between the groups in the rates of rejection or HLA mismatches as the three groups were comparable with respect to these clinical variables (Table 2). These groups were also statistically comparable with respect to the incidence of proteinuria (defined as >30 mg of protein/24 h) (Table 2). While there were differences in the incidences of class I and class II donor-specific antibodies (DSA), these were not statistically significant either taken individually or considered together.

The analyses shown in Figure 2 display the data based upon time from enrollment in this study. However, this was a cross-sectional observational trial that enrolled patients who were 1–5 years posttransplant. Thus, we also examined graft status as a function of time since transplantation for subgroups of patients consistently predicted as TOL or non-TOL (Figure 3). Interestingly, this revealed that while renal function was similar in the first  $\approx$ 1–3 years posttransplant between the two groups, it diverged thereafter with the TOL patients displaying significantly improved function compared with the non-TOL group. Thus, patients who consistently exhibited the tolerance signature since the time of study entry had superior graft function compared to those predicted non-TOL.

#### Peripheral blood B cell analyses

In previous studies, we and others have shown increased numbers of total B cells in the peripheral blood as well as increased percentages of B cells in the total lymphocyte gate (1,2,4) of spontaneously tolerance renal allograft recipients compared with those maintained on chronic immunosuppression. We have also observed a skewing within the B cell subset to naïve and transitional cells, with a reduction in the percentage of memory B cells. We used a previously described set of panels to analyze B cell subsets in our study patients at a single time point (i.e. study entry). As shown in Figure 4, we observed a significant increase in overall B cell absolute counts in patients consistently predicted TOL versus those consistently predicted as non-TOL. However, rather than having a selective increase in one or more B cell subsets, the difference in overall B cell numbers was reflected among each of the six B cell subsets we analyzed such that predicted TOL patients had higher numbers of each subset compared with predicted non-TOL individuals. Consistent with this, while the percentage of total B cells (in the lymphocyte gate) in the predicted TOL patients was significantly higher than in the predicted non-TOLs, we observed no differences in the percentage of any of the B cell subsets within the B cell gate (Figure S3).

In an earlier study of spontaneously tolerant renal transplant patients (13), we noted that elevated numbers of B



**Figure 4: B cell subsets as absolute counts between 17 consistently Predicted TOL versus 71 consistently Predicted non-TOL,  $p < 0.05$ .** B cell populations as % B cell (see Figure S3) are not statistically significant between 17 consistently Predicted TOL versus 71 consistently Predicted non-TOL. SM, switched memory; USM, unswitched memory; DN, double negative; T1 + T2, transitional B cell subsets 1 and 2; T3, transitional B cell subset 3. [https://www.itntrialshare.org/ARTIST\\_fig4.url](https://www.itntrialshare.org/ARTIST_fig4.url). TOL, tolerant.

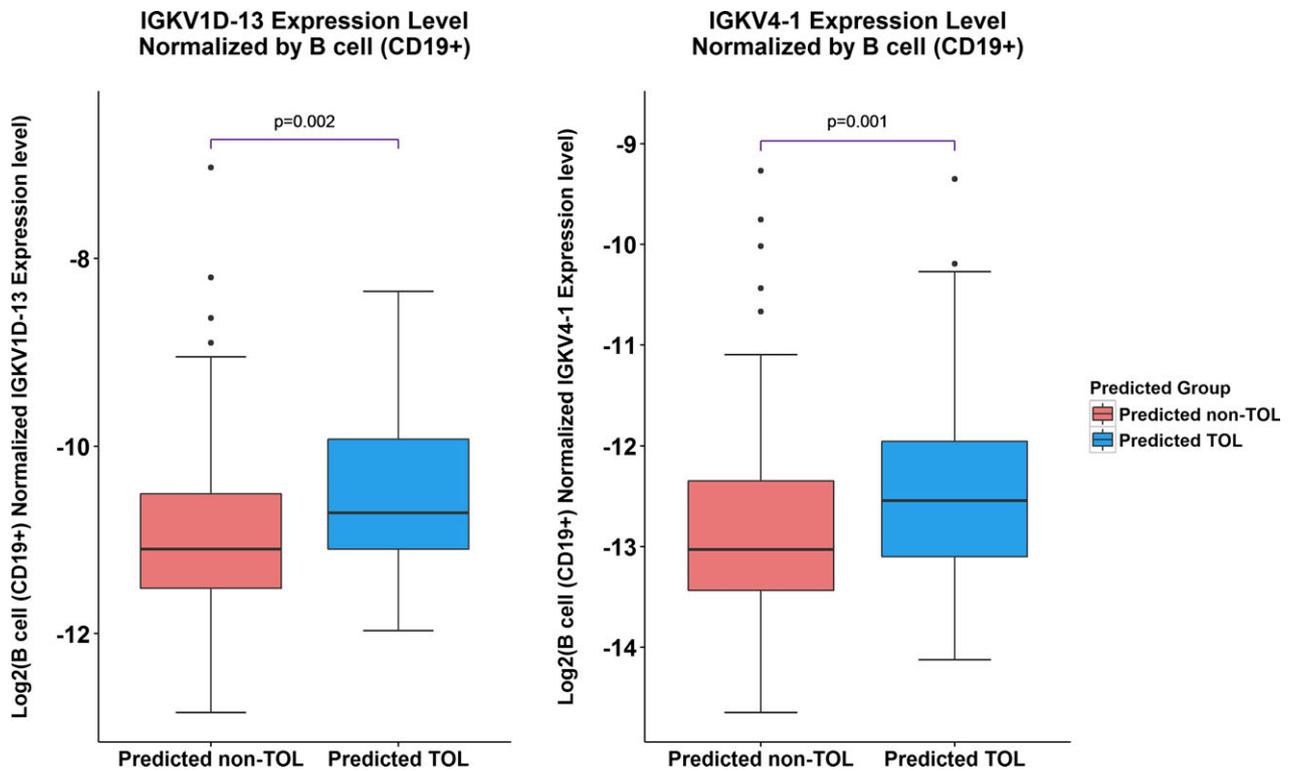
cells did not fully account for the increased transcripts of IGKV1D-13 and IGKV4-1—the B cell transcripts that form the ITN signature—as even when B cell numbers were controlled for, IGKV1D-13 and IGKV4-1 transcripts were elevated in the blood of tolerant patients. We performed that analysis in the current study and found similar results; even when normalized for B cell numbers, IGKV1D-13 and IGKV4-1 expression was significantly higher in samples at study entry that were predicted TOL compared with those predicted non-TOL (Figure 5). This increase in IGKV1D-13 and IGKV4-1 on a “per B cell” basis was also observed in the subgroup of patients consistently predicted TOL versus non-TOL, although those results did not achieve statistical significance likely due to smaller numbers of samples. Taken together, these data support the idea that our previously identified signature of tolerance is not exclusively a byproduct of elevated total B cell numbers.

#### ***Influence of immunosuppressive regimens on the prevalence of the ITN signature***

We and others have considered that a signature derived from operationally tolerant renal transplant patients might merely reflect the absence of immunosuppression, and not a tolerance process *per se*. The fact that tolerant patients often resemble healthy controls does nothing to

dispel this notion, and indeed recent work by the Indices of Tolerance consortium suggests that certain immunosuppressive agents (i.e. azathioprine and prednisone) may have selective effects on immune compartments leading to alterations in gene expression that confound tolerance signatures (14). Indeed, we noted a significant elevation in B cell counts among patients who received Campath (without an mTOR inhibitor or a CNI) compared with those who had not (Figure S4).

Thus, we analyzed the presence of the tolerance signature with whether or not the patient received induction therapy at the time of transplant and with the type of immunosuppression used at the time of study entry (Figure 6a and 6b). Examining the entire set of study participants and all sample time points, we found that TOL status was more likely to be predicted among patients who did not receive thymoglobulin as induction immunosuppression. Presence of the tolerance signature was also more likely among recipients whose immunosuppressive regimens included tacrolimus as well as those who were not receiving corticosteroids or MMF. While the number of patients receiving Campath and/or mTOR inhibitors was small, the incidence of the signature previously associated with tolerance was numerically, but not statistically, increased in recipients who received



**Figure 5: B cell gene expression normalized by CD19 absolute counts between all Predicted TOL versus All Predicted non-TOL are statistically significant.** Per cell gene expression levels between 17 consistently Predicted TOL versus 71 consistently Predicted non-TOL show a similar pattern, but are not statistically significant, likely due to the smaller number of participants (not shown). [https://www.itntrialshare.org/ARTIST\\_fig5.url](https://www.itntrialshare.org/ARTIST_fig5.url). AZA, azathioprine; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin; Tac, tacrolimus; Thymo, Thymoglobulin; TOL, tolerant.

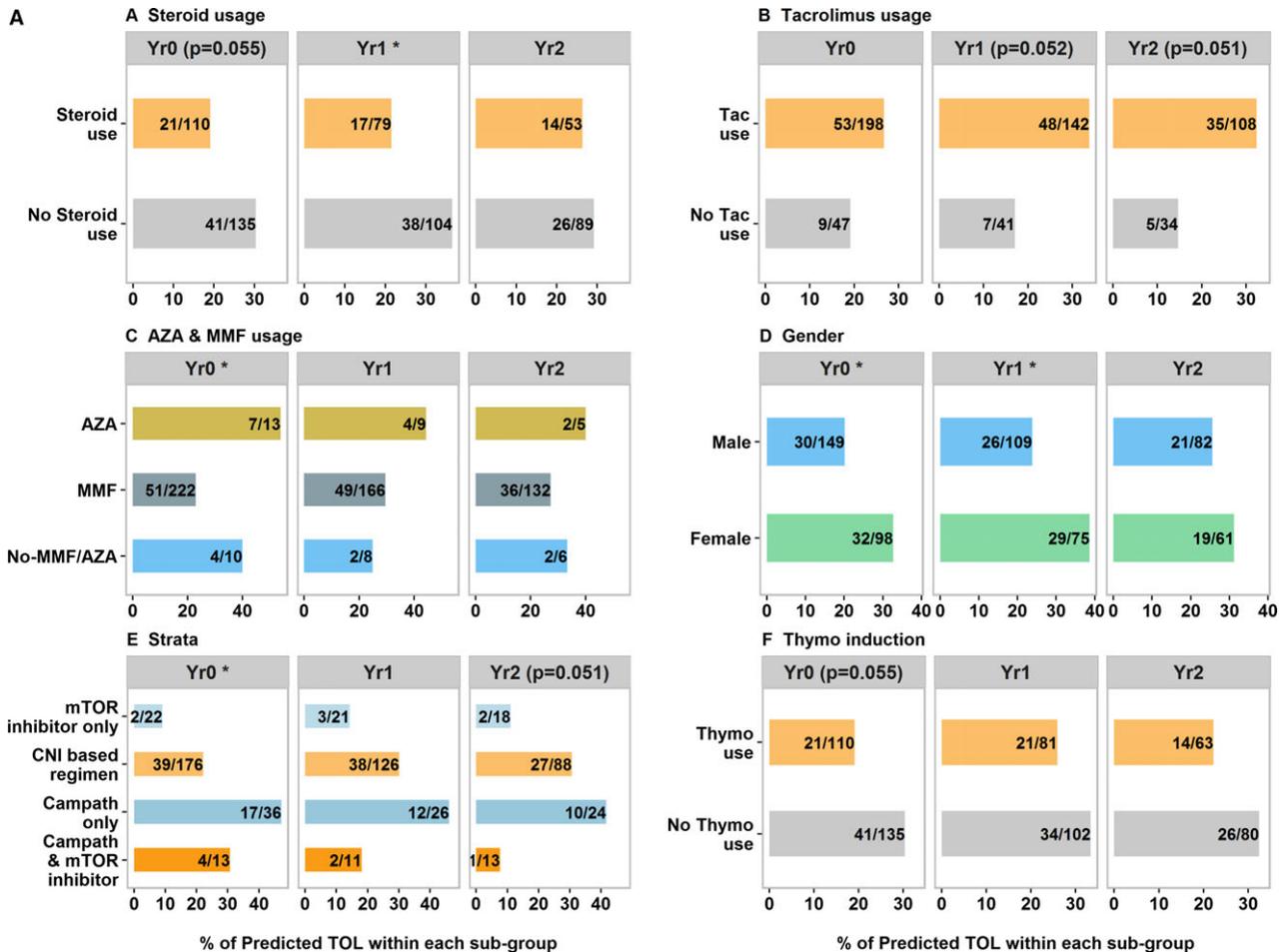
induction with Campath and reduced in those receiving an mTOR inhibitor alone or in combination with Campath. Similar considerations apply to the positive association with the tolerance signature observed in the small number of patients taking azathioprine. Finally, since, as noted above, in the majority of patients a prediction of tolerance was not stable over the time course of the study, we also analyzed the effect of immunosuppressive regimens among the subgroup of patients who were consistently predicted as TOL (Figure 5b). This subgroup exhibited the same trends seen in the entire study population.

## Discussion

Numerous examples in fields such as oncology demonstrate that the identification and validation of biological predictors of response enables personalization of care, selection of appropriately targeted therapies, and improved outcomes. While transplantation has benefited from a robust clinical database, the Scientific Registry of Transplant Recipients (SRTR), for many years, the SRTR does not capture the type of data that would support

true personalized approaches to immunosuppressive management. Consequently, changes in immunosuppression for individuals are often based upon patient demographics, clinical variables, or physician experience. Two recent studies attempting to withdrawal CNI from carefully selected recipients were both halted prematurely due to high rates of acute rejection and the *de novo* formation of DSA (8,10). These experiences highlight the need to develop biomarkers capable of better identifying those individuals in whom immunosuppressive minimization or even withdrawal is safe.

To date, most attempts to describe and validate biomarkers in kidney transplantation have focused on noninvasive determination or even prediction of acute rejection (15–19). We and others have also reported the results of studies designed to identify biomarkers associated with spontaneous tolerance following renal transplantation (1,2,4). A recent meta-analysis of five different studies confirmed that spontaneously tolerant kidney transplant recipients were characterized by relative overexpression of B cell-associated genes in the peripheral blood (20). However, each of these studies of spontaneous tolerance following kidney transplantation has been subject to



**Figure 6: Drug usage and classification.** (A) Percentage of participants Predicted TOL within clinical subgroups. Generally, no Thymo induction, no steroid use, Tacrolimus, and only Campath shows higher proportion of participants with TOL prediction. Comparisons statistically significant, \*p < 0.05, with two to three participants in the mTOR inhibitor only group, depending upon visit. Percentage of Predicted TOLs within each subgroup is shown on x-axis and proportion of Predicted TOLs is shown as bar labels. [https://www.itntrialshare.org/ARTIST\\_fig6a.url](https://www.itntrialshare.org/ARTIST_fig6a.url). (B) Proportion of 17 consistently Predicted TOL across clinical subgroups. Similar to the proportions for all TOL participants within each visit, no Thymo induction, no steroid use, Tacrolimus, and Campath only lead to higher number of participants with TOL prediction. Comparisons statistically significant, \*p < 0.05. Percentage of consistently Predicted TOLs within each subgroup is shown on x-axis and proportion of consistently Predicted TOLs is shown as bar labels. [https://www.itntrialshare.org/ARTIST\\_fig6b.url](https://www.itntrialshare.org/ARTIST_fig6b.url). mTOR, mammalian target of rapamycin; Thymo, Thymoglobulin; TOL, tolerant.

the same limitations imposed by their design. In each case, outreach was used to identify patients who had maintained stable graft function following discontinuation of immunosuppression (either due to nonadherence or as necessitated by complications of immunosuppressive therapy). Consequently, no samples were available from times at which the tolerant patients were receiving immunosuppression. Based upon this design, there is a lingering concern that the described “signatures” of tolerance could simply reflect the absence of immunosuppression. While to some observers, the similarity between the B cell expression profiles of tolerant kidney transplant recipients and healthy subjects may merely

indicate that tolerance is like normal “health,” to others it adds to this concern.

Studies conducted in the setting of liver transplantation, where the rate of spontaneous tolerance is higher and the consequences of rejection on long-term outcomes less, have also raised concerns about crafting predictable biomarkers based on studying patients only after tolerance is firmly established. Initial studies in spontaneously tolerant liver transplant recipients showed that NK cells and  $\gamma\delta$  T cells were associated with operational tolerance (21). However, subsequent studies conducted by the same group comparing

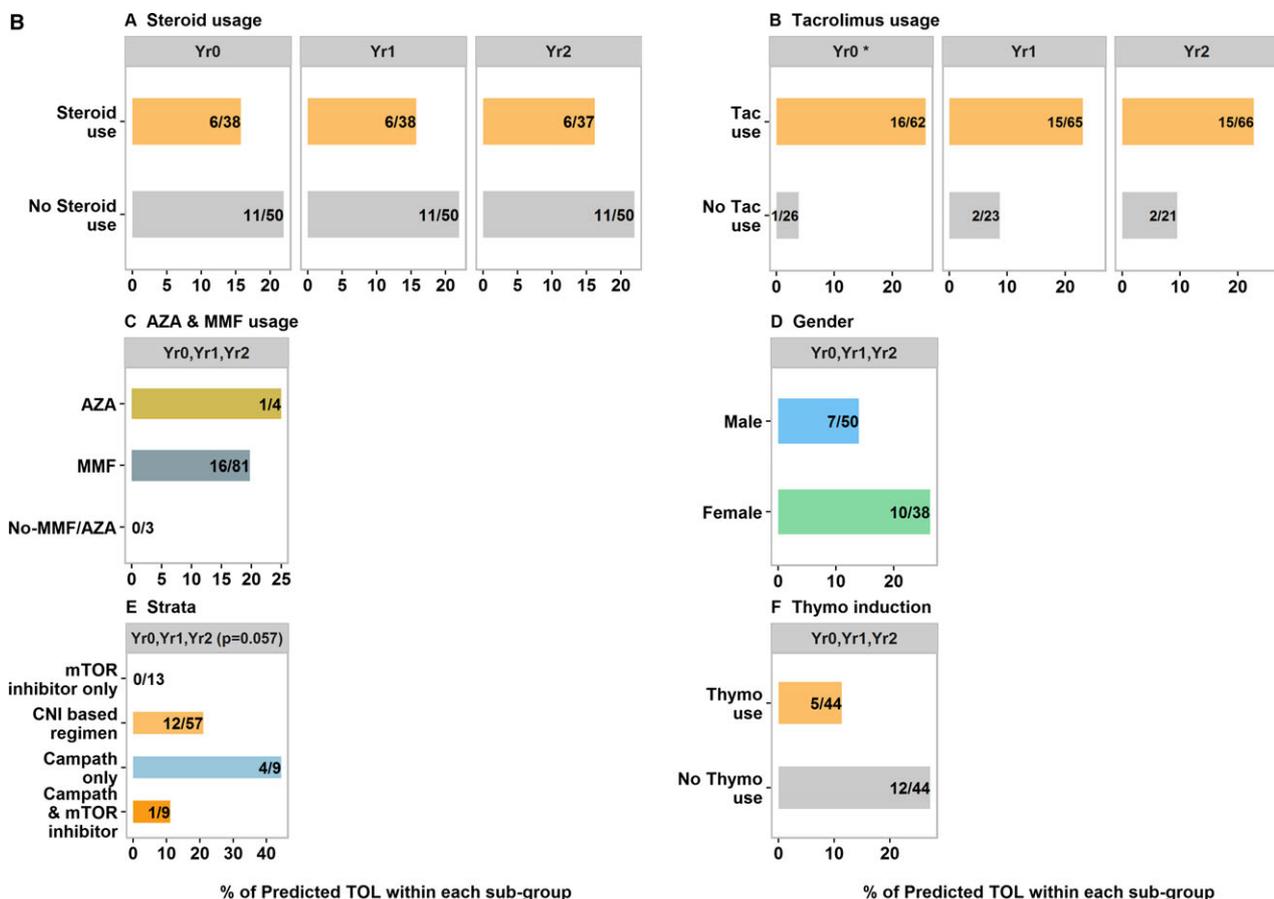


Figure 6: Continued.

patient samples from prior to and following drug withdrawal in tolerant recipients showed that these markers arose after drug withdrawal. Whether these differences in biomarkers of tolerance reflect drug effects or the evolution of tolerance mechanisms over time remain an unresolved question. However, for the purposes of identifying biomarkers to inform the management and possible reduction or cessation of immunosuppression, only those biomarkers of tolerance that are present while recipients are receiving immunosuppression will be of clinical utility.

In order to address these questions, we undertook a study with the goals of defining the prevalence of the signature in patients receiving immunosuppression who had a clinical course and level of graft function that would enable immunosuppressive minimization if a potentially predictive biomarker of tolerance was available. We believe this study has significant strengths. First, it is a prospective, multicenter trial that enrolled a large number of patients. As such, we believe the cohort of patients studied accurately reflects transplant recipient variables and transplant center practices in the United States. Second, by design the study examined recipients

treated with a number of different immunosuppressive regimens.

The primary goal of the study was to determine the prevalence of the B cell “signature of tolerance” in this population. We found that the two-gene B cell “signature” was present in 25–30% of patients at one or more time points following transplantation. More importantly, it was present in 13.7% of all those patients evaluated at each of the three study-specified time points. We believe this to be a clinically useful prevalence (i.e. frequent enough to warrant screening of subjects). Interestingly, the frequency that we observed in this study is comparable to the frequencies reported by other investigators (3.5%, 7.3%, and 11.6%, respectively) who have developed independent molecular signatures of tolerance (14,22,23).

The second aim of this study was to determine whether specific immunosuppressive agents influenced the prevalence of the two-gene signature. We predicted that the use of CNI would reduce the prevalence of a tolerance signature while mTOR inhibitors, Thymoglobulin, or alemtuzumab would increase the frequency of the signature.

In contrast, to our surprise the “signature” was observed most commonly in the cohort of recipients receiving tacrolimus. Although the numbers were small, our data suggest a trend toward less frequent detection of the two-gene “signature” in recipients who received an mTOR inhibitor or depleting induction with Thymoglobulin. Interestingly, induction with alemtuzumab was not associated with a reduced frequency of the two-gene “signature.” Finally, and consistent with a recent report, our data indicate that the signature is less common in recipients receiving prednisone (18). In the broad context, we interpret these results as further demonstration that the choice of immunosuppressive agents may influence the expression of immune response genes related specifically to B cells as well perhaps as B cell numbers and the B cell repertoire. However, we are cautious in our interpretation; not all of these trends were statistically significant. Further studies will be needed to prospectively validate these findings, and determine whether the immunosuppressive drugs affect B cell numbers and gene expression *per se* (i.e. independent of any effect on immune status towards the graft), or whether the effects on B cells are linked to alloimmunity.

Perhaps the most unexpected finding of this study is that recipients who displayed the two-gene “tolerance signature” at each of the three time points tested had significantly superior renal function as reflected by either eGFR or serum creatinine independent of immunosuppressive type. While the observed relationship between the changes in B cells and B cell–related genes and renal function remains to be more fully explored, it is interesting to consider that there may be a mechanistic relationship such that B cells with immunoregulatory/suppressive function might reduce alloimmune injury resulting in improved function and perhaps in some cases tolerance. Along this line, it is also interesting to note that immature B cells have been shown to be relatively ineffective in promoting T cell responses in murine models (24). This hypothesis, though unproven, is consistent with recent reports in experimental transplant models that tolerance is associated with increased numbers of B cells with regulatory properties that are capable of transferring tolerance as well as data from tolerant human kidney transplant recipients demonstrating that a population of B cells is capable of suppressing CD4 effector cells *in vitro* (12,25). Even absent the formal demonstration that changes in B cells and B cell–associated genes identify tolerant renal transplant recipients, the identification of B cell–related markers that identify a cohort with improved function could have important prognostic and mechanistic implications.

We acknowledge several limitations of the current work. First and foremost, the study describes the incidence of a gene expression signature that has not definitively been proven to identify tolerance following kidney transplantation. Any potential association between the

two-gene B cell “signature” and tolerance cannot be assessed in this study as no attempts were made to withdraw immunosuppression. Second, there are no data linking B cells to the improved function observed in the cohort that overexpressed the two B cell–related genes that define our previously reported signature. That said, the fact that the “signature” co-associates with the use of CNIs rather than their absence argues against the absence of drug-induced nephrotoxicity as the explanation for the improved function observed in the cohort displaying the “signature.” Finally, our previous reports on tolerant recipients demonstrated that much of the increase in B cell numbers was driven by an increase in the frequency and number of transitional and naïve B cells. In contrast, the findings of this study indicate among patients with the molecular tolerance signature, all populations of B cells are increased without the selective expansion of naïve and/or transitional B cells previously observed. The reasons for this difference remain unknown but may be related to the fact that unlike the patients reported in the current study, previously studied transplant recipients who displayed the “signature” had well-established tolerance that in most cases had persisted for years.

At this point the question remains as to how to more definitively determine the meaning of the “signature” we have described as well as how it might be used in the clinical care of kidney transplant recipients. As a next step, the data we report in this article may provide enough information to undertake a cautious study of immunosuppressive drug minimization. Given the degree of overlap between numbers of B cells (Figure 2) and gene expression corrected for B cell number (Figure 5), the monitoring data described in the current article are not by themselves a sufficient basis on which to taper immunosuppressive medications. In addition, while we processed our samples at a single time point to eliminate a “batch effect,” this might not be feasible in a clinical trial and thus assay standardization will be important. Taken together, this means that the design of such a trial, the magnitude of the attempted immunosuppressive drug minimization, and the safety measures to be employed all remain topics for further careful deliberation.

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## Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

## Disclosure

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

## References

1. Newell KA, Asare A, Kirk AD, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest* 2010; 120: 1836–1847.
2. Sagoo P, Perucha E, Sawitzki B, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. *J Clin Invest* 2010; 120: 1848–1861.
3. Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci USA* 2007; 104: 15448–15453.
4. Pallier A, Hillion S, Danger R, et al. Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. *Kidney Int* 2010; 78: 503–513.
5. Leventhal J, Abecassis M, Miller J, et al. Tolerance induction in HLA disparate living donor kidney transplantation by donor stem cell infusion: Durable chimerism predicts outcome. *Transplantation* 2013; 95: 169–176.
6. Kawai T, Sachs DH, Sprangers B, et al. Long-term results in recipients of combined HLA-mismatched kidney and bone marrow transplantation without maintenance immunosuppression. *Am J Transplant* 2014; 14: 1599–1611.
7. Scandling JD, Busque S, Shizuru JA, et al. Chimerism, graft survival, and withdrawal of immunosuppressive drugs in HLA matched and mismatched patients after living donor kidney and hematopoietic cell transplantation. *Am J Transplant* 2015; 15: 695–704.
8. Hricik DE, Formica RN, Nickerson P, et al. Adverse outcomes of tacrolimus withdrawal in immune-quiet kidney transplant recipients. *J Am Soc Nephrol* 2015; 26: 3114–3122.
9. Kirk AD, Guasch A, Xu H, et al. Renal transplantation using belatacept without maintenance steroids or calcineurin inhibitors. *Am J Transplant* 2014; 14: 1142–1151.
10. Dugast E, Soullillou JP, Foucher Y, et al. Failure of calcineurin inhibitor (tacrolimus) weaning randomized trial in long-term stable kidney transplant recipients. *Am J Transplant* 2016; 16: 3255–3261.
11. Chesneau M, Pallier A, Braza F, et al. Unique B cell differentiation profile in tolerant kidney transplant patients. *Am J Transplant* 2014; 14: 144–155.
12. 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.
13. Newell KA, Asare A, Sanz I, et al. Longitudinal studies of a B cell-derived signature of tolerance in renal transplant recipients. *Am J Transplant* 2015; 15: 2908–2920.
14. Rebollo-Mesa I, Nova-Lamperti E, Mobillo P, et al. Biomarkers of tolerance in kidney transplantation: Are we predicting tolerance or response to immunosuppressive treatment? *Am J Transplant* 2016; 16: 3443–3457.
15. Suthanthiran M, Schwartz JE, Ding R, et al. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. *N Engl J Med* 2013; 369: 20–31.
16. Roedder S, Sigdel T, Salomonis N, et al. The kSORT assay to detect renal transplant patients at high risk for acute rejection: Results of the multicenter AART study. *PLoS Med* 2014; 11: e1001759.
17. Kurian SM, Williams AN, Gelbart T, et al. Molecular classifiers for acute kidney transplant rejection in peripheral blood by whole genome gene expression profiling. *Am J Transplant* 2014; 14: 1164–1172.
18. Reeve J, Chang J, Salazar ID, Lopez MM, Halloran PF. Using molecular phenotyping to guide improvements in the histologic diagnosis of T cell-mediated rejection. *Am J Transplant* 2016; 16: 1183–1192.
19. Hricik DE, Nickerson P, Formica RN, et al. Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury. *Am J Transplant* 2013; 13: 2634–2644.
20. Baron D, Ramstein G, Chesneau M, et al. A common gene signature across multiple studies relate biomarkers and functional regulation in tolerance to renal allograft. *Kidney Int* 2015; 87: 984–995.
21. Martinez-Llordella M, Lozano JJ, Puig-Pey I, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest* 2008; 118: 2845–2857.
22. Brouard S, Le Bars A, Dufay A, et al. Identification of a gene expression profile associated with operational tolerance among a selected group of stable kidney transplant patients. *Transpl Int* 2011; 24: 536–547.
23. Roedder S, Li L, Alonso MN, et al. A three-gene assay for monitoring immune quiescence in kidney transplantation. *J Am Soc Nephrol* 2015; 26: 2042–2053.
24. Chung JB, Wells AD, Adler S, Jacob A, Turka LA, Monroe JG. Incomplete activation of CD4 T cells by antigen-presenting transitional immature B cells: Implications for peripheral B and T cell responsiveness. *J Immunol* 2003; 171: 1758–1767.
25. Durand J, Huchet V, Merieau E, et al. Regulatory B cells with a partial defect in CD40 signaling and overexpressing granzyme B transfer allograft tolerance in rodents. *J Immunol* 2015; 195: 5035–5044.

## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Figure S1:** Consort diagram of sample comparisons tested. [https://www.itntrialshare.org/ARTIST\\_figS1.url](https://www.itntrialshare.org/ARTIST_figS1.url).

**Figure S2:** Predictive classification of participants with probability cut-off of 0.5. Seventeen patients consistently Predicted TOL versus 71 consistently Predicted non-TOL. [https://www.itntrialshare.org/ARTIST\\_figS3.url](https://www.itntrialshare.org/ARTIST_figS3.url).

**Figure S3:** B cell subsets as % B cells between 17 consistently Predicted TOL versus 71 consistently Predicted non-

TOL. Other than Total B cells, B cell subpopulations differences as % B cell are not statistically significant. [https://www.itntrialshare.org/ARTIST\\_figS2.url](https://www.itntrialshare.org/ARTIST_figS2.url).

**Figure S4:** Differences in B cell counts between different drug regimens for all participants. [https://www.itntrialshare.org/ARTIST\\_figS4.url](https://www.itntrialshare.org/ARTIST_figS4.url).