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Biomarkers of Operational Tolerance Following Kidney Transplantation – The Immune Tolerance Network Studies of Spontaneously Tolerant Kidney Transplant Recipients

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Abstract

Studies of kidney transplant recipients who have developed spontaneous and sustained tolerance have revealed an association with B cells. Unexpectedly tolerant individuals are characterized by increased numbers and frequencies of B cells in the blood and increased expression of genes associated with B cells in the blood and urine. Comparisons of the B cell repertoires of tolerant individuals and those receiving immunosuppression reveal that not only are the B cells more numerous but developmental differences result in a repertoire comprised of more naïve and transitional B cells in the tolerant cohort. B cells isolated from tolerant individuals also display functional differences compared to those from individuals receiving immunosuppression. Many of these differences may serve to suppress alloimmunity. Lastly a significant number of transplant recipients receiving standard immunosuppression display B cell-biased patterns of gene expression predictive of tolerance or a pro-tolerogenic state. Interestingly, this pattern is associated with improved renal allograft function. While recent studies have raised the concern that immunosuppressive drugs heavily influence B cell-based “signatures of tolerance”, a substantial body of work suggests that differences in B cells may be a useful tool for identifying tolerant kidney transplant recipients or guiding their immunosuppressive management.

Keywords

Transplantation; Kidney; Tolerance; Biomarkers; B cells

Introduction

Spontaneous tolerance following kidney transplantation in humans, as opposed to tolerance intentionally induced by a specific treatment regimen, is not a newly observed phenomenon. As early as 1975 a small series of patients who had stopped immunosuppression and not acutely rejected was reported (1). Although two of the six patients ultimately experienced

acute rejection, the authors concluded that once immunosuppression was stopped, unless rejection occurred it was not necessary to resume immunosuppressive therapy. However, a subsequent report of a larger number kidney transplant recipients displaying spontaneous tolerance emphasized the high frequency of acute rejection and subsequent graft loss and urged resumption of immunosuppression with the possible exception of those who had maintained stable function for greater than three years after stopping all immunosuppression (2). Concerns about the wisdom of pursuing tolerance to transplanted kidneys are far from resolved. Even recently authorities in the field of kidney transplantation have voiced new concerns about the safety and long-term outcomes of complete immunosuppressive drug withdrawal in kidney transplant recipients (3). These reports highlight the fact that tolerance in the clinical setting as opposed to the laboratory is considered to be operational. Operational tolerance is defined as the persistence of normal function in the absence of immunosuppression. The study of tolerance is hampered by the absence of validated assays or biomarkers capable of confirming the existence of robust donor-specific unresponsiveness. Furthermore, there are currently no biomarkers capable of determining how robust or long lasting a state of operational tolerance may be. This absence of validated biomarkers of tolerance is a significant barrier to the study of tolerance in the clinic, the immunosuppressive management of patients receiving little or no immunosuppression, and the weaning of immunosuppression. Two recent reports describing studies attempting to wean calcineurin inhibitors from patients predicted to be at a low risk of rejection on the basis of clinical characteristics (absence of DSA, stable graft function, biopsies without evidence of inflammation) demonstrate the challenges of immunosuppressive drug minimization in stable kidney transplant recipients. Both studies were stopped prematurely due to high rates of rejection and/or the formation of DSA following attempted drug withdrawal (4, 5).

Goals, Design, and Limitations of the ITN Studies of Spontaneously Tolerant Kidney Transplant Recipients

Unlike studies of tolerance following liver transplantation where the rates of operational tolerance are significantly higher than kidney (6) and the long-term consequences of rejection following immunosuppressive drug reduction or withdrawal limited with the prompt diagnosis and reintroduction of more intensive immunosuppression (7), it is generally thought that spontaneous tolerance following kidney transplantation is a rare event and that episodes of rejection associated with drug withdrawal likely to compromise long-term graft function and survival. Thus in the absence of validated biomarkers of operational tolerance most in the field believe it is unsafe to intentionally withdraw immunosuppression unless prompted by a clinical indication. Realizing that there were rare patients who had ceased all immunosuppression and continued to display stable, good function of the transplanted kidney and had thus already assumed the risk of drug withdrawal of their own volition we chose a study design that sought to identify kidney transplant recipients who had previously stopped all immunosuppression. Identified patients who agreed to participate provided demographic and clinical data as well as biological samples for mechanistic assays. When feasible, almost exclusively in the setting of living donor kidney transplantation, efforts were made to also obtain donor cells for additional mechanistic assays. Following

enrollment subjects underwent testing to assess renal function (serum creatinine and calculation of eGFR), allograft injury (proteinuria and allograft biopsy), alloimmunity (cellular assays of immunity and screening for DSA), and more general studies to determine the phenotype of peripheral blood cells by flow cytometry as well as gene expression profiles of peripheral blood cells (gene array and QT-PCR) and shed urinary epithelial cells (QT-PCR). Data and biological samples were obtained from several additional cohorts for the purpose of comparison.

At the outset it should be emphasized that several elements of the study design created perceived or actual limitations in with respect to the studies' conclusions. The first potential limitation arises from the absence of a true control group. Unlike studies of tolerance performed in the laboratory where it is possible to design a control group that mimics the experimental group in all meaningful variables aside from the therapy used to induce tolerance or the tolerant state itself, this is not feasible in the clinical setting. The importance of the comparison group chosen is illustrated by the findings of Brouard et al. (8). In this group's seminal study of gene expression profiles in spontaneously tolerant kidney transplant recipients they chose to use subjects with chronic rejection, which they defined as immune-mediated kidney allograft failure with return to dialysis and cessation of immunosuppression as their primary control group. This choice likely contributes to differences between many of the findings in this study and subsequent studies by this or other groups where the primary comparison of tolerant subjects was to those with stable renal allograft function receiving conventional immunosuppression. In designing the ITN study protocol several comparison groups were considered. Indeed numerous cohorts that could be considered as an appropriate comparison for one or more variables were enrolled including subjects with stable function while receiving conventional immunosuppression, subjects receiving conventional immunosuppression who on the basis of clinical features and biopsy findings were determined to have alloimmune-mediated graft injury, patients with stable function while receiving corticosteroid monotherapy, recipients of kidneys from an identical twin donor, and healthy volunteers.

We chose as our primary comparison group kidney transplant recipients receiving conventional immunosuppression who had good and stable graft function. This choice was based on our goal of identifying a signature of tolerance to transplanted kidneys that could be used as a tool to facilitate the safer minimization or complete withdrawal of immunosuppression in the clinical setting. We reasoned that patients already experiencing significant graft dysfunction or those with significant infectious or neoplastic conditions would not likely be candidates of protocol guided management of immunosuppression but would be managed based on other more pressing clinical considerations. However, our choice of comparing tolerant patients to those receiving ongoing immunosuppression raised the very real concern that we may be measuring a signature of the absence of immunosuppression. Consistent with this concern two groups (9, 10) as well as our own data to be discussed later demonstrate that the choice of immunosuppressive agents can influence the prevalence of B cells, a factor associated with spontaneous tolerance to transplanted kidneys in numerous studies. While the impact of immunosuppressive drugs on the prevalence of a tolerance signature derived from the comparison of tolerant patients to those receiving any immunosuppression remains a concern, two factors suggest that the described

B cell-based tolerance signatures are not solely related to the effects of immunosuppressive agents. Firstly, as discussed later a not inconsequential proportion of patients receiving immunosuppression are consistently predicted to be tolerant based on an increase in the number of B cells or an increased expression of B cell-related genes. Secondly, comparing tolerant liver transplant recipients to those receiving immunosuppression fails to demonstrate the changes in B cells and B cell-related genes that characterize spontaneous tolerance following kidney transplantation (11). Together these findings suggest that the absence of immunosuppression alone is not responsible for the B cell-related changes that have been associated with spontaneous tolerance to transplanted kidneys.

A second concern directly related to the study design arises from the enrollment of patients who already display the tolerant phenotype rather than enrolling patients prior to the development of tolerance. This becomes a concern if the mechanisms responsible for tolerance evolve and change over time as first proposed by the late Charley Orosz (12). In this case determining biomarkers in patients with established tolerance may detect biomarkers reflective of mechanisms that maintain the tolerant state but are perhaps distinct from the mechanisms contributing to the initial development of tolerance. This is possibility is supported by the findings that the cell populations associated with the development and maintenance of tolerance following liver transplantation differed in samples obtained prior to and following the weaning of immunosuppression (13). Similarly, initial reports describing immunologic differences between tolerant and non-tolerant participants in the Massachusetts General Hospital tolerance trials reported that at early time points regulatory T cells were enriched in the blood and allografts of tolerant subjects (14). At later time points differences in regulatory cell frequency between the groups disappeared at the same time as donor alloantigen specific T cells were deleted from the repertoire (15).

The final design element that influences the interpretation of our studies is the absence of biopsy data. Although the initial protocol included allograft biopsies at the time of the first study visit, the protocol was modified based on an adverse event early in the study in which a protocol biopsy resulted in hematuria, acute kidney injury, and a small arteriovenous fistula that resolved spontaneously. The absence of allograft tissue precludes histologic assessment for factors such as subclinical inflammation or causes of allograft injury distinct from alloimmunity (recurrent disease, drug toxicity, infections, etc.). In a study of operationally tolerant kidney transplant recipients Brouard et al. noted that among the 27 originally tolerant individuals 13 had a functional graft without evidence of sensitization to the donor (DSA), six had a functional allograft with evidence of donor sensitization, and eight experienced graft loss due to a mixture of alloimmune and non-alloimmune causes (16). Obviously the inability to distinguish between declining function or graft loss caused by alloimmune and non-alloimmune causes would be important when considering how accurately biomarkers of tolerance predict the persistence of tolerance. In addition without allograft tissue it is not possible to interrogate the allograft itself with respect to immunologic processes that may be occurring in the transplanted organ. This is potentially very important as some groups have found that assessment of immune processes occurring within the transplanted organ are more informative than those detected in the blood (13).

Summary of findings from the ITN Studies of Spontaneously Tolerant Kidney Transplant Recipients

Enrollment and clinical features of tolerant recipients in ITN studies (Table 1)

Since 2004 our studies have identified 39 individuals displaying spontaneous operational tolerance following kidney transplantation. As a result of missing information or inadequate sample quality seven individuals were not included in any analysis. Key demographic and clinical data for the 32 analyzed individuals is shown in Table 1. Notably these individuals were virtually all Caucasian, received well matched kidneys from living donors, ceased immunosuppression as a consequence of non-adherence, and maintained very good renal function at an average of 15 years following complete cessation of immunosuppression. Their clinical course following transplantation is notable for very low rates of both acute cellular rejection and humoral sensitization. It is apparent that the demographic and clinical features of these tolerant individuals are clearly not typical of the general population undergoing kidney transplantation. As a group individuals developing tolerance were at low immunologic risk and received high quality, well-matched kidneys from living donors. They experienced very low rates of alloimmune events and maintained very good allograft function for years following transplantation and cessation of immunosuppression.

Initial findings from mechanistic assays performed in spontaneously tolerant kidney transplant recipients

In considering which assays to include in our study of tolerant kidney transplant recipients we hypothesized that tolerance would develop and be maintained by an increase in the number or activity of regulatory T cells. Consequently there was an initial emphasis on determining the phenotype of T cells in blood and the development of cell based assays of alloimmunity. However, assays such as the CFSE mixed lymphocyte reaction and the ELISPOT proved challenging in terms of the cell numbers required, technical reproducibility, and their correlation with the clinical phenotype.

Gene expression studies—A comparison of data generated using microarrays demonstrated that only 30 genes displayed a 2-fold difference in expression between tolerant individuals and those receiving standard immunosuppression (17). To our surprise 22 of these genes were specific to B cells as defined by the OMIM database including many genes involved in B cell activation and differentiation. In order to develop a more quantitative approach multiplex real-time PCR was used to analyze the expression of 228 genes selected based on the findings of the microarray studies or their known roles in immunity and tolerance. Using this approach 31 genes were identified that differentiated tolerant individuals from those receiving standard immunosuppression including 17 of the genes originally identified by microarray. Consistent with the findings of the analysis performed using microarrays 26 of the genes identified by real-time PCR were B cell specific. Additional analyses to determine a smaller group of classifier genes capable of distinguishing tolerant individuals from those being treated with standard immunosuppression identified three genes, *IGKV4-1, IGLL1*, and *IGKV1D-13*, that categorized tolerant and non-tolerant individuals in training and test sets with both a high

positive and negative predictive value. It is important to note that no differences in gene expression were noted between tolerant kidney transplant recipients and healthy volunteers. While this could be interpreted as a consequence of the absence of immunosuppressive agents in both groups, it could alternatively suggest that tolerant transplant recipients have acquired or returned to the more normal equilibrium of the immune system seen in healthy individuals. Further evidence suggesting a role for B cells in tolerance to transplanted kidneys arose from studies of gene expression profiles in the urinary sediment cells. The expression of 18 genes postulated to be important in tolerance or alloimmunity was determined in urinary sediment cells by quantitative real-time PCR. The only gene differentially expressed between tolerant individuals and the cohort receiving immunosuppression was CD20, which was significantly higher in the tolerant group. This is an interesting and potentially important observation because as already mentioned the properties of immune cells within the transplanted organ are thought to be more informative in some situations that those in the blood.

Flow cytometric immunophenotyping—Consistent with the findings of the gene expression profiling immunophenotyping of whole blood showed increased frequencies and numbers of total B cells (17). Significant changes between tolerant individuals and kidney transplant recipients receiving standard immunosuppression were not observed for other lymphocyte or mononuclear cell populations. Interestingly much of the increase in B cells was related to increased numbers of naïve and transitional B cells. This finding was reproduced using PBMCs from kidney transplant recipients participating in the Indices of Tolerance (IOT) study in Europe (18). As transitional B cells with regulatory or suppressive properties had been described in murine models of autoimmunity we chose to examine the functional properties of transitional B cells. Because IL-10 production is the dominant characteristic of murine regulatory B cells, we compared the production in vitro of IL-10 by transitional B cells from tolerant kidney transplant recipients to that of transitional B cells from recipients receiving standard immunosuppression. Consistent with a possible role for regulatory B cells in spontaneous tolerance to transplanted kidneys transitional B cells from tolerant subjects produced more IL-10, but not TGFβ, than did transitional B cells from recipients receiving standard immunosuppression. Further evidence from the IOT study supporting a role of B cells in spontaneous tolerance arose from microarray studies of peripheral blood that demonstrated increased expression of B cell-related genes as well as molecular pathways associated with B cells in tolerant kidney transplant recipients.

Further refinement of the “B cell signature” of tolerance to transplanted kidneys – Insights from FACTOR

FACTOR, the second ITN-sponsored study of spontaneously tolerant kidney transplant recipients was designed to follow up on our original finding that tolerant recipients differed from those receiving immunosuppression in terms of both B cell number and phenotype (19). The main aim of this study was to assess the stability of the “B cell signature” of tolerance over time. We reasoned that if changes in the B cell number, frequency, or phenotype distinguished tolerant kidney transplant recipients from those receiving immunosuppression, the observed changes should be stable over time. Disappearance of the “B cell signature” without a concomitant loss of the tolerant phenotype would call into

question the relevance of the original observation. To this end the expression of the two most predictive genes, IGKV1D-13 and IGLL-1, from the original 3-gene descriptor were analyzed at four time points each separated by one year. As shown in Figure 1 (figure 6 AJT paper 2) the expression of both genes was stable over the time period of the study in the tolerant cohort. However, the statistical significance of the difference between the expression of these genes diminished over time from enrollment or transplantation due to increased expression in the cohort receiving standard immunosuppression. Acknowledging that due to sample availability not all individuals were studied at each time point this observation is interesting in two regards. First, as previously noted the incidence of spontaneous tolerance following liver transplantation increases with time following transplantation (6). This suggests that at least in some individuals mechanisms capable of promoting tolerance or at least suppressing alloimmunity develop over time. It is possible that this same phenomenon may be occurring over time following kidney transplantation as reflected by increased expression of IGKV1D-13 and IGLL-1 in the cohort of patients receiving standard immunosuppression. The second observation pertains to concerns that the immunosuppressive agents themselves or their absence are the predominate factor influencing B cells and the putative “B cell-based tolerance signature”. As the cohort of patients in the standard immunosuppression group were on average more than five years out from their transplant at the time of enrollment and had stable kidney graft function, it seems highly unlikely that for the group as a whole there were significant changes in their immunosuppressive regimens. Thus, the increased prevalence of the signature in patients receiving standard immunosuppression seems unlikely to be associated with changes in immunosuppression and argues that the presence of immunosuppressive drugs does not prevent acquisition of the “B cell signature of tolerance” or conversely that the absence of immunosuppression is not a prerequisite for developing the changes in B cells associated with spontaneous tolerance.

Another observation arising from the FACTOR study is that in addition to tolerant patients having increased numbers of B cells compared to recipients receiving immunosuppression, the B cells themselves appear phenotypically different. When compared to recipients receiving immunosuppression, tolerant individuals displayed increased frequencies of T1 and T2 (but not T3) transitional B cells and lower frequencies of switched and unswitched memory B cells. As shown in Figure 2 (Figure 3 AJT paper 2) the phenotypic composition of the B cell repertoire correlated better with clinical phenotype (tolerance versus standard immunosuppression) than did the absolute B cell number. Furthermore, when normalized for CD19+ cells IGKV1D-13 was increased on a per cell basis in tolerant individuals as when compared to those receiving immunosuppression.

The last objective of the FACTOR study was to re-examine the three gene “B cell-based tolerance signature” derived from our earlier study. Using a different PCR methodology our data confirmed that the two most predictive genes, IGKV1D-13 and IGLL-1, were still strongly associated with tolerance using samples from both previously studied subjects and newly enrolled tolerant subjects. The third gene, IGKV4-1 that had the weakest association with tolerance in our original analysis, was no longer associated with the tolerant phenotype when re-analyzed in the FACTOR study. In addition to confirming the association of IGKV1D-13 and IGLL-1 with operational tolerance in subjects from the original tolerant

cohort as well as a new cohort of five tolerant kidney transplant recipients we examined the expression of IGKV1D-13 in kidney transplant recipients undergoing weaning of immunosuppression as part of another ITN study (ITN013) (20) or recipients who were rendered tolerant as part of ITN sponsored studies of combined kidney and bone marrow transplantation (ITN010 and ITN036) (21, 22). This analysis showed that patients successfully weaned to sirolimus monotherapy and those rendered tolerant as a result of a protocol combining kidney and bone marrow transplantation displayed increased expression of IGKV1D-13 that was comparable to the increase we had previously observed in spontaneously tolerant kidney transplant recipients. It is also noteworthy that the one patient in the combined kidney and bone marrow transplant protocol who developed early rejection and had immunosuppression reinstituted expressed a level of IGKV1D-13 that was comparable to those of subjects in our ITN registry who were receiving conventional immunosuppression.

The B cell “Tolerance Signature” in kidney transplant recipients receiving immunosuppression – the ARTIST study

ARTIST was conceived as the next step toward considering the use of the B cell “Signature of Tolerance” clinically as one factor guiding decisions about immunosuppressive drug minimization or withdrawal. We reasoned that in order to be useful the signature would need to be present in a large enough fraction of patients receiving conventional immunosuppression to be worthwhile and not simply a hunt for “a needle in a haystack” but not so frequent as to suggest the inclusion of a large number of patients with no predisposition toward tolerance. Estimates of the percentage of kidney transplant recipients receiving immunosuppression who might actually be tolerant based on tolerance biomarkers reported by other groups ranged from 3.5% to 12% (9, 23, 24). In our original analysis we noted that 13.3% of the 30 subjects receiving IS were categorized as tolerant base on the three gene “signature”. By way of comparison 16.7% of the 30 subjects receiving CNI-based standard immunosuppression were predicted to be tolerant using the B cell-based tolerance signature developed by the IOT study group (18). The aim of ARTIST was to study a much larger number of subjects with the intentional inclusion of subjects receiving different maintenance immunosuppressive regimens (25). To this end as we had previously shown that that IGKV4-1 did not add to the sensitivity or specificity of identifying tolerant patients in FACTOR, we examined the prevalence over a two year period of the two genes most closely associated with operational tolerance, IGKV1D-13 and IGKV4-1, in 248 kidney transplant recipients between 1 and 5 years post-transplant who with stable renal function and had been free from rejection for the year proceeding enrollment. At any single time point 25 – 30% of patients were classified as tolerant based upon the levels of expression of IGKV1D-13 and IGKV4-1. Evaluable samples at all three time points were available for 124 patients. Using more stringent criteria, the consistent classification as tolerant at each of the three study visits, 13.7% of individuals were classified as tolerant while 71 patients were consistently classified as non-tolerant. Not surprisingly as the “classification” of potentially tolerant was based on the increased expression of two B cell restricted genes flow phenotyping of PBMC showed increased numbers of B cells in the cohort predicted to be tolerant. Unlike our previous analysis rather than being the result of a preferential expansion of transitional and naïve B cells, all B cell subsets were increased in those receiving

maintenance immunosuppression but predicted to be tolerant. Most interestingly, when we compared the renal function of those consistently predicted to be tolerant versus those consistently predicted to be non-tolerant we noted improved function in the cohort predicted to be tolerant. Correcting for time since transplant we observed that although the groups classified as predicted tolerant and predicted non-tolerant had equivalent renal function initially, over time the renal function of those predicted to be tolerant increased markedly while the renal function of those predicted non-tolerant remained stable or declined slightly (Figure 3 ARTIST AJT paper).

The final aim of ARTIST was to determine the impact of various immunosuppressive agents on the prevalence of the tolerance signature. Contrary to initial expectations the frequency of predicted tolerance was greatest in patients receiving a CNI-based maintenance immunosuppressive regimen and reduced in those receiving corticosteroids, mycophenolate mofetil, or induction with Thymoglobulin. Whether these associations reflect direct effects of the agents on B cells themselves and are independent of a pro-tolerogenic effect of B cells or promote changes in B cells that favor a reduction in the overall alloimmune response remains to be determined. In this vein it is interesting to note that despite increased B cells in the cohort of subjects predicted to be tolerant, none of the evaluable patients had DSA in contrast to a 16.9% incidence of DSA in the cohort consistently predicted to be non-tolerant. Taken as a whole these data suggest that increased numbers of B cells that do not produce DSA are associated with improved renal function and are not inconsistent with a role for suppressive or immunoregulatory B cells in transplantation and tolerance.

A potential role for cell populations other than B cells in spontaneous tolerance to transplanted kidneys

While the majority of analyses of spontaneously tolerant kidney transplant recipients reveal an association with increased numbers of B cells, other analyses have reported changes in different populations of immune cells such as dendritic cells to be associated with tolerance (24). A separate analysis of tolerant kidney transplant recipients enrolled in the ITN study used the *trans-vivo* delayed type hypersensitivity (DTH) assay to determine the relative contributions of T effector and regulatory cells recognizing alloantigens through the indirect pathway (26). This analysis found that the ratio of regulatory to effector T cell responses to donor antigens was increased in tolerant kidney transplant recipients relative to those with stable function who were receiving conventional immunosuppression. This response was dependent upon TGFβ but not IL-10 and was also independent of B cells. These data provide additional support for the hypothesis that spontaneous tolerance following kidney transplantation may be the result of multiple mechanisms working simultaneously to suppress anti-donor immune responses.

Consideration of how the ITN Studies Compare to Other Studies of Tolerant Kidney Transplant Recipients

While the similarities and differences between our findings and those of others studying tolerance to transplanted kidneys will be apparent upon a complete reading of this supplement, for convenience we have highlighted a few of the most notable comparisons.

First, as noted the vast majority of studies examining spontaneously tolerant recipients of transplanted kidneys report that they are characterized by increased numbers of B cells (17–19, 27). A formal meta-analysis of samples provided by the three major study groups (the ITN, RISET, and investigators at the University of Nantes) identified a robust signature of tolerance comprised of 20 genes with the majority related to B cells but including genes related to CD4 T cells as well as the inhibition of CD14 monocytes (28). This composite 20 gene signature correctly classified subjects as tolerant in 91.7% of cases. Using these datasets a composite score comprised of molecular and clinical variables has recently been described (29). The composite score is based upon the expression of six genes that are primarily related to B cells and two clinical factors (subject age at sample acquisition and age at transplantation). A second point to note with regard to the association of B cells and spontaneous tolerance following kidney transplantation is that a recent study suggesting that most of the previously described B cell-based tolerance signatures were significantly influenced by the immunosuppressive agents themselves (or their absence), found that of the nine genes reported to constitute a tolerance signature that is independent of immunosuppression two were related to B cells, one to B cells and T cells, one to T cells alone, and one to macrophages (9). Thus while these data may call into question specific components of B cell-based tolerance signatures, they do not directly challenge previous findings that B cells are associated with spontaneous tolerance to transplanted kidneys.

In contrast to the relative preponderance of literature supporting an association between B cells and spontaneous tolerance, the limited data available do not suggest an association between B cells and tolerance achieved through intentional tolerance protocols involving lymphodepletion, non-myeloablative conditioning, and combined kidney and hematopoietic cell transplantation (30). As mechanistic assays performed on subjects undergoing transplantation using regimens designed to induce tolerance will be thoroughly addressed in other manuscripts included in this special issue only a brief recap will be provided here. Studies in HLA haploidentical living donor and recipient pairs at Stanford University and in non-HLA matched living donor pairs at Northwestern University have not yet identified biomarkers that consistently distinguish between subjects who develop tolerance and those that do not. Studies performed in HLA identical living donor pairs at Northwestern University revealed increased numbers of T and B cells for periods up to five years following conditioning and transplantation but these increases were comparable in those that did and did not develop tolerance suggesting that these changes are not useful as biomarkers of tolerance or mechanistically related to the development of tolerance (30). As already mentioned studies of haploidentical living donor pairs undergoing a regimen consisting of lymphodepletion, conditioning, and combined bone marrow and kidney transplantation as part of an ITN-sponsored study conducted at the Massachusetts General Hospital did show an increase in B cells numbers similar to those observed in spontaneously tolerant kidney transplant recipients but also showed evidence of early expansion of regulatory T cells in the transplanted kidney with clonal deletion of alloreactive T cell clones at later time points (14, 15). Understanding the relative contributions of each of these mechanisms to the development and maintenance of tolerance may be highly valuable for monitoring kidney transplant recipients for the development of tolerance as well as potentially aiding in the design and conduct of future protocols intended to induce tolerance.

Future Directions

Over the past decade numerous groups have independently and collaboratively demonstrated an association between increased numbers of B cells, a shift in the B cell repertoire toward naïve and transitional B cells, and an increase in the expression of B cell-related genes to the state of spontaneously arising operational tolerance. Many of these same groups have shown that the various “tolerance signatures” occur with a non-trivial frequency in kidney transplant recipients receiving maintenance immunosuppression. It would seem that the next step would be to design and conduct trials of immunosuppressive drug minimization or withdrawal in those expressing the signature. However, numerous concerns persist that give pause to those considering this approach. First, it remains possible that the currently reported signatures of tolerance are an artifact reflecting the absence of immunosuppression or at least the absence of specific immunosuppressive agents. Although we agree that different immunosuppressive agents have different effects on B cells and hence most of the putative “tolerance signatures”, we believe a significant amount of data support the role of B cells in spontaneous tolerance to transplanted kidneys. These data include not only the association of changes in B cells with established spontaneous tolerance following kidney transplantation but also several reports, both clinical and experimental, demonstrating functional properties of B cells isolated from tolerant recipients capable of suppressing alloimmunity (31–33). However, given the potentially long-lasting and devastating consequences of unleashing alloimmune responses on the long-term function and survival of transplanted kidneys it is uncertain whether or not these data are sufficient to overcome the safety concerns of investigators working in the field of transplantation tolerance.

Conclusion

The ITN study of individuals who spontaneously developed tolerance following kidney transplantation has uncovered an association between B cells and tolerance. Unexpectedly the number of B cells and the expression of B cell-associated genes is increased in tolerant recipients with respect to kidney transplant recipients maintained on conventional immunosuppression. Accompanying this increase in B cells are changes in the B cell repertoire with a shift toward phenotypically less mature cells. While these findings are common to studies conducted by a number of groups, it remains unclear if or how they could be applied to the clinical management of kidney transplant recipients. At a minimum it would seem that a more detailed understanding of the functional properties of the expanded B cells in tolerant individuals and the development of more robust assays to detect emerging alloimmune responses before they are well established and mediate allograft injury that may not be fully reversible will be critical prior to undertaking trials designed to use these types of biomarkers for the withdrawal of immunosuppressive agents.

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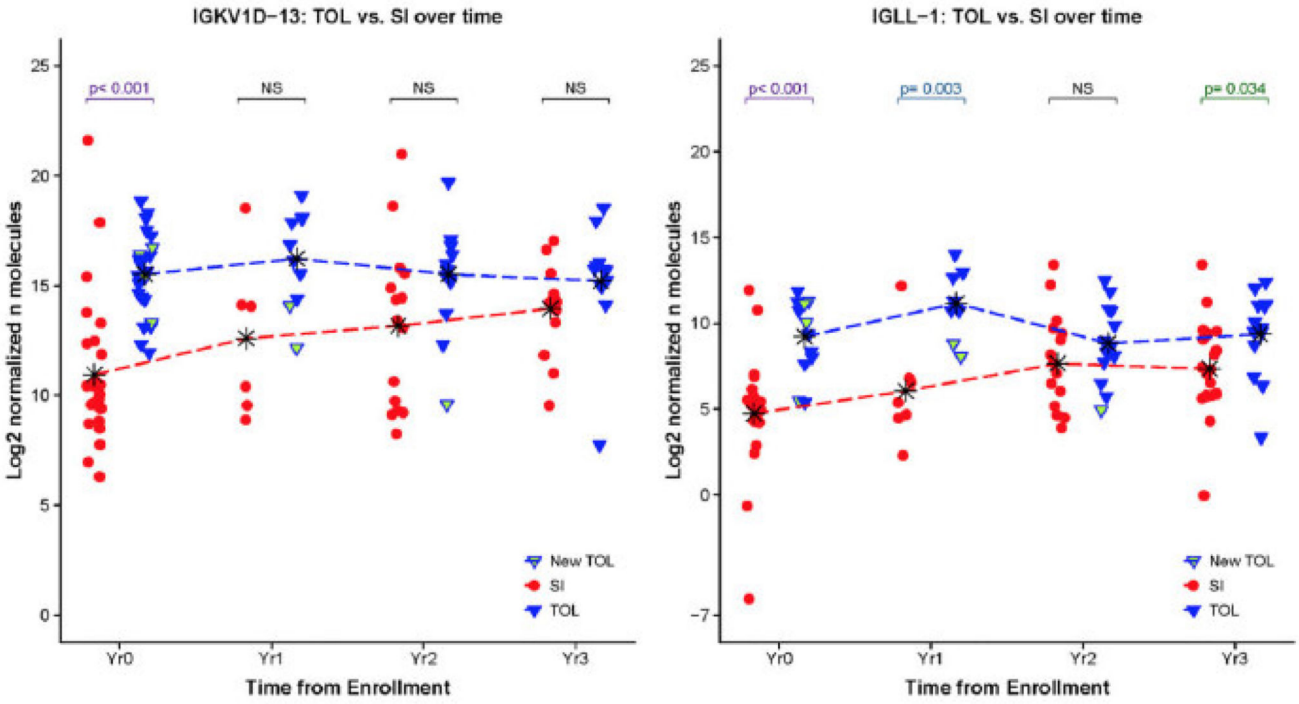

Figure 1.

Changes in the expression of IGKV1D-13 and IGLL-1 over time. Scatter plots showing the normalized values for the expression of IGKV1D-13 and IGLL-1 in blood as a function of the time of each subject's enrollment in the study. Mean values are denoted by an * and connected by dashed lines.

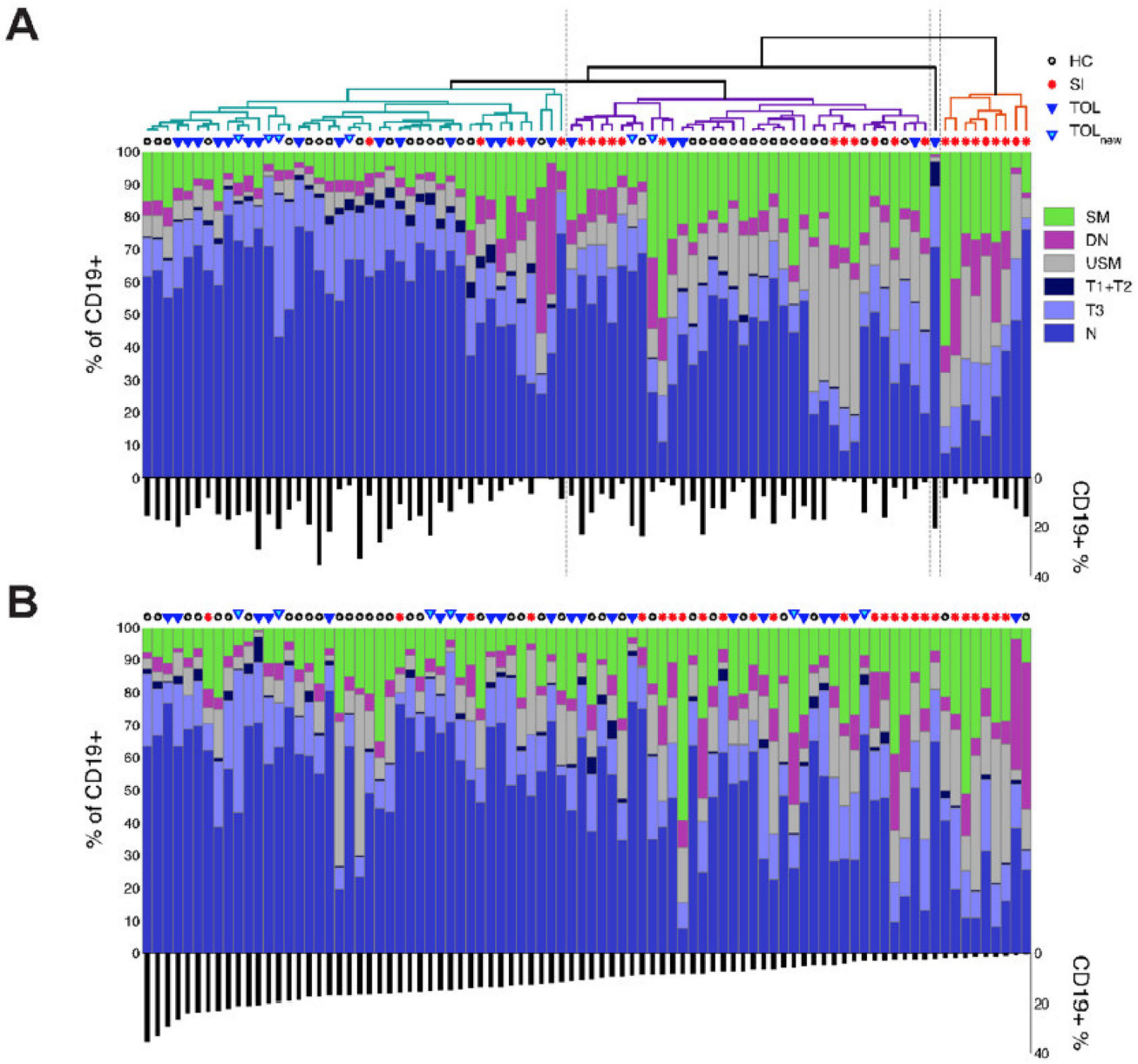

Figure 2.

Composition of the B cell repertoire for tolerant kidney transplant recipients, those receiving standard immunosuppression, and healthy volunteers. Panel A shows the relative frequencies of six subsets of B cells is shown as a function of an unsupervised, hierarchical clustering of B cell profiles. The various colors correspond to the proportion of the indicated B cell subset relative to the total population of CD19+ cells. In panel B the individual subjects are arranged sequentially based on the total number of CD19+ cells as shown along the base of the X axis.

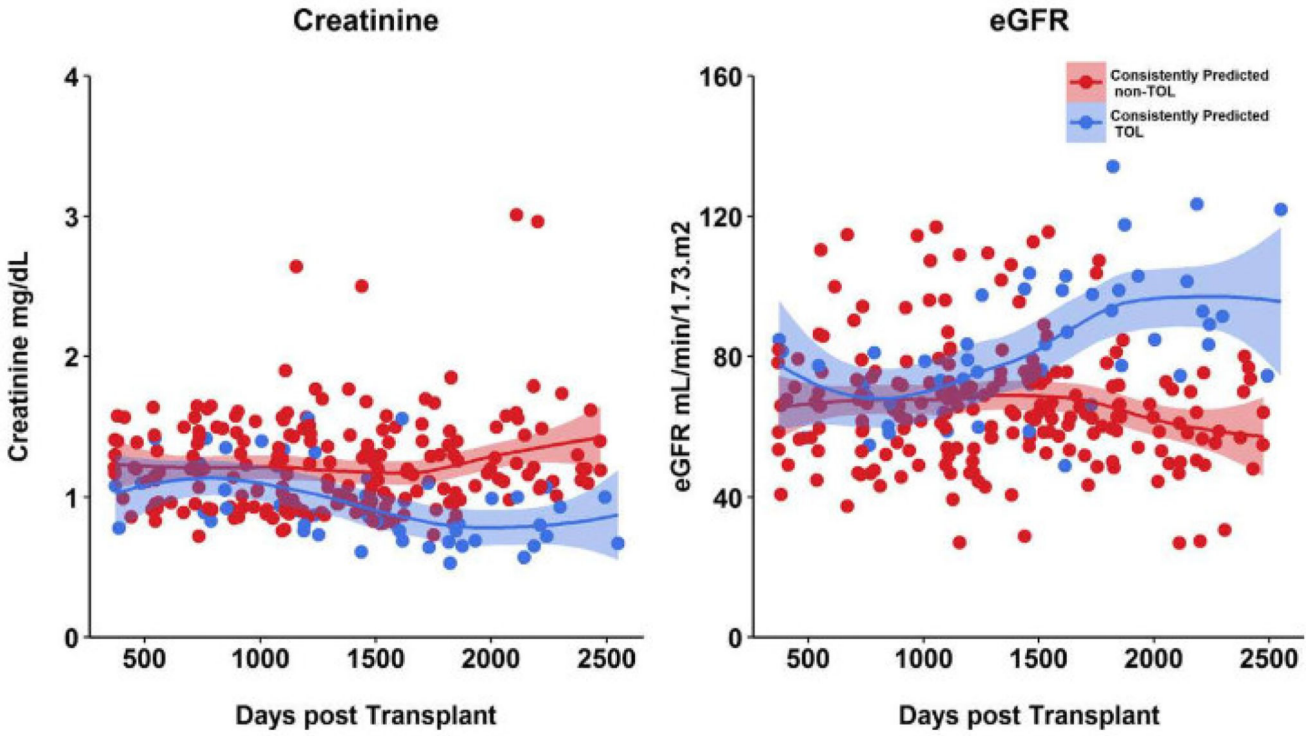

Figure 3.

Comparison of renal function for subjects consistently predicted to be tolerant versus those consistently predicted not to be tolerant. Renal function at the indicated time point following transplantation as assessed by creatinine and eGFR for 17 subjects receiving conventional immunosuppression that were consistently predicted to be tolerant based on their expression of IGKV1D-13 and IGLL-1 versus 71 subjects consistently predicted not to be tolerant.

Table I

Demographic and Clinical Characteristics of Spontaneously Tolerant Kidney Transplant Recipients

	TOL (n=32)
Race, *n*	
Asian	1
Black or African American	
White	31
American Indian or Alaska Native	
Ethnicity, *n*	
Hispanic or Latino	3
Not Hispanic or Latino	29
Gender, *n*	
Female	14
Male	18
Donor type, *n*	
Living-related	21
Living-unrelated	4
Deceased donor	5
Data missing	2
Age at Enrollment, yrs, mean (SD)	53 (10.7)
Age at Transplantation, yrs, mean (SD)	32 (12.2)
Interval between transplant and enrollment, yrs, mean (SD)	21 (9.8)
Donor Age, yrs, mean (SD)	52 (8.7)
Primary cause for renal failure[1], *n*	
Genetic	3
Diabetes mellitus	3
Etiology uncertain	
HIV nephropathy	
Hypertension	
Immune mediated	14
Pyelonephritis/interstitial nephritis	1
Structural	3
Other	10
HLA mismatch (A, B, and DR loci)[2]	
mean (SD)	1.1 (1.67)
Data missing, *n*	10
Years off IS	
yrs, mean (SD)	15 (11.4)
Data missing, *n*	4

	TOL (n=32)
Reason for Discontinuing IS, *n*	
Medical condition	3
Non-compliance	23
Data missing	6
Documented episodes of acute rejection, *n*	
No acute rejection	29
Mild acute cellular rejection (Grade IA)	2
Mild acute cellular rejection (Grade IB)	
Moderate acute cellular rejection (Grade IIA)	1
Moderate acute cellular rejection (Grade IIB)	
Renal Function, mean (SD)	
Creatinine level, mg/dl	1.5 (1.58)
Proteinuria (>30 mg/dl), *n*	
< 30 mg/24hrs	
>=30 mg/24hrs	26
Data missing	6

[1]Two ITN507 TOL had multiple primary causes for renal failure

[2]Allele or antigen level mismatch analysis was performed based on the resolution of available HLA data. Synonymous mutations were not considered mismatches.